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METABOLISM OF MERCURY COMPOUNDS IN MICROORGANISMS

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This report describes the physiology and ecology of mercury-resistant and mercury-metabolizing bacteria from Chesapeake Bay. Evidence is presented which establishes a role for bacteria in the cycling of mercury in the estuarine environment. From the results of a survey of Hg° production among a group of randomly selected, $HgCl_2$-resistant bacteria and mixed natural microbial populations, it was established that the enumeration of mercury-resistant bacteria by plate counting is a valid index of potential Hg^{2+} metabolism *in situ*.

The distribution of mercury-resistant bacteria was significantly different in water and sediment, from station to station, and seasonally; the proportion of Hg^{2+}-resistant bacteria among the total, viable, heterotrophic bacterial population reached a reproducible maximum in Spring and was positively correlated with water turbidity, dissolved oxygen concentration, and mercury concentration in the sediment.

These findings and the observation of the evolution of Hg° from freshly collected water and sediment suggest that bacteria may contribute substantially to the mobilization and transformation of mercury from existing deposits in Chesapeake Bay, specifically, and in the aquatic environment, in general.

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METABOLISM OF MERCURY COMPOUNDS IN MICROORGANISMS

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ABSTRACT

This report describes the physiology and ecology of mercury-resistant, mercury-metabolizing bacteria from Chesapeake Bay. Evidence is presented which establishes a role for bacteria in the cycling of mercury in the estuarine environment.

Mercury-resistant aerobic, heterotrophic bacteria were isolated from water and sediment at six stations, representing various levels of environmental quality, in upper Chesapeake Bay. These organisms were found to be resistant to an array of inorganic and organic mercury compounds and heavy metal ions. It was also observed that in individual cultures demonstrating resistance to $HgCl_2$ and phenylmercuric acetate, the resistance was adaptive and was also related to metabolic capability for degrading the compounds to elemental mercury (Hg^o). However, cultures in the process of adaptation evidenced delayed growth and cell division, which were dependent upon mercury concentration. The cultures also developed a variety of morphological irregularities associated with cell wall and cytoplasmic membrane synthesis and function.

From the results of a survey of Hg^o production among a group of randomly selected, $HgCl_2$-resistant bacteria and mixed natural microbial populations, it was established that the enumeration of mercury-resistant bacteria by plate counting is a valid index of potential Hg^{2+} metabolism *in situ*.

The population of mercury-resistant bacteria, primarily *Pseudomonas* spp., varied quantitatively in time and from station to station. The distribution of mercury-resistant bacteria was significantly different in water and sediment, from station to station, and seasonally. Yet, the proportion of Hg^{2+} resistant bacteria among the total, viable, heterotrophic bacterial population reached a reproducible maximum in spring and was positively correlated with water turbidity, dissolved oxygen concentration, and mercury concentration in the sediment.

These findings and the observation of the evolution of Hg^o from freshly collected water and sediment suggest that bacteria may contribute substantially to the mobilization and transformation of mercury from existing deposits in Chesapeake Bay, specifically, and in the aquatic environment, in general.

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SECTION I

CONCLUSIONS

The results of this investigation prove that aerobic, heterotrophic bacteria can be influential in the mobilization of mercury from existing resources in Chesapeake Bay.

Mercury-resistant bacteria are numerous throughout Chesapeake Bay in water and sediment, but are most prevalent in those areas where high total mercury levels are found in sediment. Bacteria "adapt" to mercury, in terms of total numbers and generic distribution in a given location and in the ability to resist and reduce mercury, presented in the form of mercuric and phenylmercuric ions, to the elemental state. The former adaptation probably represents a long-term, selective effect, and the latter a more rapid, or transient, microbial response to mercury contamination. Although bacteria adapt successfully, in vitro studies suggest that bacteria can undergo cell damage in the presence of excessive levels of mercury contamination. Bacterial growth occurs at a threshold inhibitory concentration of mercury, with the appearance of numerous morphological effects, which indicate cell wall and cytoplasmic membrane damage.

The proportion of mercury-resistant bacteria in the total viable, aerobic, heterotrophic bacterial population serves as a valid index of potential mercury mobilization in Chesapeake Bay water and sediment. Using this criterion, it can be concluded that bacterial mobilization of mercury, through the formation of Hg^{o}, varies seasonally and is related to water turbidity, water dissolved oxygen, and total mercury concentrations in sediment. A reproducible peak in activity occurs in Spring. This phenomenon can have serious consequences, in terms of effects on life cycles of higher marine organisms, and the rates of accumulation of mercury through marine and estuarine food webs.

SECTION II

RECOMMENDATIONS

We have identified and characterized a group of bacteria which are potentially significant in the mobilization and transformation of mercury in Chesapeake Bay. Our results and the reports of others indicate that Hg is continually mobilized from existing deposits of mercury in the inshore marine and estuarine environments. As mercury levels did not decrease significantly during the 17 months in which we monitored the several stations in Chesapeake Bay, which were included in our study, we assume that processes of addition and mobilization maintain a steady state level of mercury. A mercury budget should be calculated to determine whether production of Hg^{o} correlates with theoretical seasonal fluctuations predicted from our results. Clearly a "microbial mobilization index" (MMI) will then prove to be a useful parameter for assessing the environmental impact of presently existing or new reservoirs of mercury.

The fate of Hg^{o}, following release into the aqueous medium, should also be investigated to determine: 1) the proportion of Hg lost from the environment through volatilization; and 2) the chemical species and ultimate destination of mercury in solution under <u>in situ</u> conditions. This information must eventually be obtained in order to establish the sources and routes of mercury in marine and estuarine food webs.

Finally, our findings underline the importance of an understanding of the microbial population structure in the natural environment. An unequivocal conclusion from our studies is that the bacteria play a significant role in the cycling and introduction of mercury and other pollutants to the marine and estuarine food webs. More attention must be placed on understanding and managing the bacteria, fungi, and other microorganisms in the environment.

SECTION III

INTRODUCTION

Mercury and its compounds (organic and inorganic) are known to undergo a number of chemical and biological transformations. Microorganisms, in particular bacteria, have been documented as agents in the formation of methyl mercury and elemental mercury from inorganic mercury. Similarly, bacteria have been shown to be capable of degrading PMA, MeHg and other organomercurials to Hg^{o} and simple carbon compounds.

Inorganic mercury in complexation with organic material is felt to be the predominant form of mercury in the estuarine environment. We and other investigators have observed the evolution of Hg^{o} from samples of water and sediment. Based upon these observations and the known metabolic capabilities of bacteria, we hypothesized a role for bacteria in the transformations and mobilization of mercury in Chesapeake Bay. This report describes our investigation of the physiological and ecological properties of mercury-resistant bacteria. It also presents evidence for the relationship of bacterial mercury resistance and metabolism.

SECTION IV

PHYSIOLOGICAL AND CULTURAL CHARACTERISTICS OF MERCURY-RESISTANT BACTERIA

INTRODUCTION

The major objective of this research project was to determine the mechanism and extent of influence of bacteria in the transformations of mercury which take place in the estuarine environment. Isolated reports by other investigators (cited in later sections) of bacterial synthesis and metabolism of mercury compounds stimulated the initiation of the work summarized in this report. The first major premise was that bacterial resistance to mercury is a function of ability to detoxify mercury by metabolic transformation. A corollary was that measurement of numbers of mercury-resistant bacteria *in situ* should provide an index of real, or potential, microbiological mercury transformation in Chesapeake Bay. The following section is a description of the experimental plan followed in the characterization and isolation of mercury-resistant bacteria from Chesapeake Bay.

MATERIALS AND METHODS

Isolation, Culture and Identification of Bacteria

Media and methods are described elsewhere (Materials and Methods, Section V).

Mercury Analysis

Samples of sediment were allowed to settle, supernatant liquids, if any, were removed, and the sediments were air dried. Unfiltered samples of water were acidified to 0.5 N HNO_3, and plankton samples were freeze dried. All samples were wet ashed and analyzed for total mercury by flameless atomic absorption according to "EPA Provisional Method for Mercury in Sediment and Water," January 1972. The vapor phase analyses of PMA-metabolizing bacteria are described elsewhere (1). Six plates of basal medium containing 0.3 ppm of PMA were inoculated with cultures grown on slants of the same medium. The cultures were placed in a hermetically sealed glass container provided with an on-line dual beam atomic absorption spectrophotometer. Six uninoculated plates served as

controls. Samples of the vapor phase were analyzed for benzene using a flow splitter for simultaneous thermocouple and flame ionization detection. Samples were injected under the following conditions: isothermal at 50 C, N_2 carrier flow at 30 ml/min, 10% Apieson L on 80/100 Supelcoport in 0.225 in by 6 ft. stainless steel columns. Samples were calibrated against 2.0 ml benzene-air mixtures from which cross-integration of thermocouple and flame ionization detector peak areas yielded a limit of detection of 0.01 ppm for benzene in air.

Radio Isotope Experiments

^{203}Hg-labeled $HgCl_2$ or PMA were added to suspensions of cells in basal broth or 0.01 M phosphate or TRIS-buffered (pH 7) "three salts" solutions and the suspensions were incubated with shaking or agitation with a magnetic spin bar or aerated through capillary tubes. Samples were removed and added directly to liquid scintillation cocktail or filtered through 0.45 micron Millipore filters. The filters were washed with three 1 ml volumes of salts and placed in scintillation cocktail to assess cell-bound activity. Corrections for chemical quenching and decay of the isotope were applied.

RESULTS AND DISCUSSION

Determinants of Bacterial Mercury Metabolism

Resistance of natural populations of aerobic, heterotrophic bacteria was measured by ability to form colonies on a simple solid growth medium supplemented with selected inorganic and organic mercury compounds. The preparation and incubation of the medium was varied.

Total mercury analysis of natural materials provides only limited information with respect to chemical form and biologically available concentrations of mercury in a microenvironment. Consequently, mercury concentrations used in our selective media were arbitrarily chosen initially. Samples of water, sediment, and homogenized plankton were routinely spread on agar containing 6 ppm $HgCl_2$ (22.1 microgram atoms of Hg/liter) or 3 ppm phenylmercuric acetate (PMA) (3.9 microgram atoms of Hg/liter). PMA was found to be significantly more toxic on a per-mole-of-Hg basis than $HgCl_2$ and was subsequently reduced in concentration to 0.3 ppm. The media were prepared by addition of either aqueous solutions of $HgCl_2$ in sterile "three salts" solution or alcoholic solutions of PMA to the sterile, molten agar medium. It was found that sterilization of the solutions by membrane filtration was unnecessary and caused a reduction in final mercury concentration of the solutions.

The concentration of resistant bacteria in each sample was expressed as a percent of the "total viable, aerobic, heterotrophic bacterial count" (TVC) obtained by counting colonies appearing after 7 days incubation at 25 C. The comparative effects of selected concentrations of mercury and concentrations approximating environmental levels are shown in Table 1. Levels as low as 1.2 ppb of PMA or 1.2 ppb of $HgCl_2$ showed measurable

effects upon bacterial populations. It is logical to assume that concentrations of mercury actually encountered in the environment should induce detectable effects in the corresponding bacterial flora.

Table 1. COMPARISON OF RESISTANCE OF SEDIMENT AND WATER POPULATIONS TO DIFFERENT LEVELS OF $HgCl_2$ AND PMA

Station	Test medium	Percent of TVC[a] comprising mercury resistant bacteria	
		Water	Sediment
Patuxent R. mouth 5/15/72	6 ppm $HgCl_2$	0.2	2.6
	0.12 ppm $HgCl_2$	-	75.3
	1.2 ppb $HgCl_2$	72.0	-
	3 ppm PMA	< 0.09	0.05
	0.12 ppm PMA	-	3.7
	1.2 ppb PMA	48.3	-
Potomac R. mouth 5/16/72	6 ppm $HgCl_2$	9.4	0.76
	0.12 ppm $HgCl_2$	-	93.4
	3 ppm PMA	< 0.1	< 0.005
	0.12 ppm PMA	-	2.5
	1.2 ppb PMA	19.2	-

[a]TVC = total viable, aerobic, heterotrophic bacterial population. (Reproduced with the permission of the Marine Technology Society.)

The effect of incubation temperature on numbers of mercury-resistant bacteria is shown in Table 2. As incubation temperature was increased to 37 C, apparent mercury resistance increased greater than ten-fold. This result may have been a consequence of increased volatilization of the mercury from the medium or a selection for mercury-resistant populations. The known non-biological reduction of mercuric ion which occurs in this medium (see Section VI) tends to favor the former explanation. At the temperature (15 C) most closely approximating the *in situ* temperature, the largest TVC was obtained.

Spangler *et al.* (2) have reported that mercury-resistant, methyl mercury-degrading bacteria metabolize methyl mercury to methane and elemental mercury (Hg^0) relatively more extensively under anaerobic rather than aerobic conditions. Consequently, plates of $HgCl_2$ medium were inoculated

with dilutions of water and sediment and incubated aerobically and anaerobically, for comparison. The percent of mercury-resistant bacteria was always lower for samples taken from three different locations when plates were incubated anaerobically (Table 3). These findings are not consistent with properties of methyl mercury-metabolizing bacteria as published by others, but they do agree with those of mercuric ion-reducing bacteria reported later in this section.

Table 2. EFFECT OF INCUBATION TEMPERATURE ON BACTERIAL VIABLE COUNT AND RESISTANCE TO $HgCl_2$

Incubation temperature (C)	Incubation time (days)	Total viable count (x 10^6/gram)	Percent resistant[b]
37	7	1.45	16.7
25	7	2.60	7.8
15	14	18.80	1.4
2	14	11.70	1.1

[a]A sample of sediment (B2-5/24/73) was diluted and plated on basal medium, with and without 6 ppm of $HgCl_2$. Surface water temperature was 16.9 C when the sample was taken.

[b]Percentage of the TVC resistant to 6 ppm of $HgCl_2$.

Table 3. COMPARATIVE EFFECTS OF AEROBIC AND ANAEROBIC INCUBATION ON BACTERIAL RESISTANCE TO $HgCl_2$

Sample	Percent resistant[a]	
	Aerobic incubation	Anaerobic incubation[b]
B2 - 5/24/73	7.0	1.0
A2 - 5/24/73	1.3	0.3
EB1 - 5/25/73	1.8	0.3

[a]Samples of sediment were diluted and spread on basal medium agar, with and without 6 ppm $HgCl_2$, and incubated for 7 days at 25 C.

[b]Incubated in BioQuest (Cockeysville, Maryland) anaerobic jars containing CO_2-enriched anaerobic atmosphere produced by the Gas Pak (BioQuest).

Since an estuary is subjected to extensive changes in salinity, among other parameters, the effects of the salt content of the growth medium used for routine isolation of mercury-resistant bacteria was evaluated for samples taken from sites encompassing a wide range of salinities (Table 4). Media of three salinities, including the routine isolation medium were compared. Media of the highest (26.59 °/oo) and lowest (2.66 °/oo) salinities consistently yielded higher proportions of mercury-resistant bacteria than the medium in general use in our studies (11.38 °/oo). The effects of salt concentration may be related to a salt requirement for mercury metabolism (this Section), or to selective effects on the bacterial populations in the samples tested. The extreme effects of salt observed in the case of samples of lowest salinity (B2-5/15/72) suggested that the latter argument should also be considered.

Table 4. EFFECT OF SALT CONCENTRATION ON BACTERIAL RESISTANCE TO $HgCl_2$

Sample	Salinity of surface water (°/oo)	Salinity of growth medium[a] (°/oo)	Percent resistant[b] Water	Sediment
B1 - 1/18/72	5.94	2.66	7.60	22.90
		11.38[c]	7.90	16.40
		26.59	17.00	19.50
EB1 - 1/31/72	10.56	2.66	—	0.79
		11.38	—	0.08
		26.59	—	0.74
B1 - 5/15/72	1.56	2.66	12.90	11.50
		11.38	4.20	8.20
		26.59	50.00	29.20
York R. mouth	16.40	2.66	5.30	6.30
		11.38	0.50	3.50
		26.59	0.16	5.90
B2 - 5/24/73	5.60	2.66	—	9.25
		11.38	—	7.80
		26.59	—	11.10

[a]Samples of water and sediment were spread on basal medium containing one of three concentrations of artificial sea water (NaCl : $MgCl_2 \cdot 6H_2O$: KCl = 100 : 23 : 3) with and without 6 ppm of $HgCl_2$ added.

[b]Percent of total viable, heterotrophic bacterial population capable of growth in medium containing 6 ppm of $HgCl_2$ after 7 days at 25 C.

[c]Concentration of salts routinely used in the medium for assay of mercury resistance.

Characteristics of Mercury-Resistant Bacteria

To define the natural habitats of mercury-resistant bacteria, water and sediment samples were separated into filterable, planktonic, and interstitial fractions, respectively (Table 5). Results showed that a non-uniform distribution of both mercury-resistant bacteria and total mercury concentrations existed among water, plankton, and sediment samples. The relative enrichment, i.e., greater concentrations, of mercury in sediments and living organisms, in comparison to water, is consistent with other published data (3, 4) and agrees with the hypothesis that planktonic forms of life may be influential in the transport of mercury, as well as introduction of mercury, into food chains. A definite trend in the data was that mercury-resistant bacterial populations found associated with plankton were relatively larger than those for water or sediment. Observations of water and sediments indicated that in most cases, mercury-resistant bacteria were also distributed non-uniformly between different particle size fractions. This suggests that an examination of mercury levels and bacterial populations of individual microenvironments would be the most logical approach to the problem of defining the relationship of mercury-resistant bacterial population size to environmental mercury levels.

A variety of bacterial cultures were isolated and characterized during the initial 2 years of the investigation. Fig. 1 shows the average comparative population distributions of $HgCl_2$ resistant and total populations of bacteria. These distributions were obtained by sampling and testing cultures randomly selected from count plates with and without added $HgCl_2$. The relatively greater diversity of the total population, with respect to the generic categories used, and the relative enrichment for Pseudomonas species among the resistant population is evident.

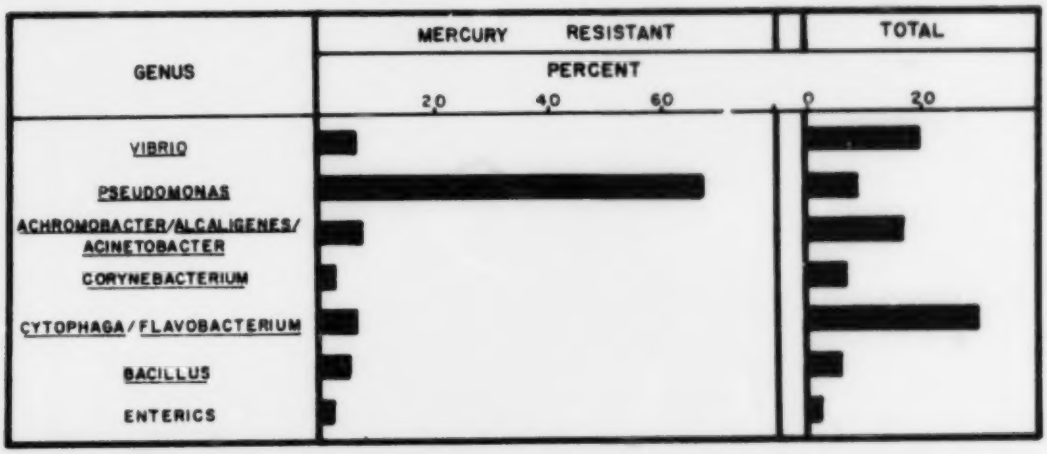

Figure 1. Average distribution of genera in total and $HgCl_2$ resistant populations. (Reproduced with the permission of the Marine Technology Society.)

Table 5. DISTRIBUTION OF BACTERIAL $HgCl_2$ RESISTANCE[a] AND TOTAL MERCURY CONCENTRATIONS AMONG WATER AND SEDIMENT FRACTIONS

	Source										
	Surface water			Filtered water[b]	Surface sediment			Interstitial water[c]	Plankton[d]		
Sample[e]	% Resistant $HgCl_2$	% Resistant PMA	[Hg] (ppb)	% Resistant $HgCl_2$	% Resistant $HgCl_2$	% Resistant PMA	[Hg] (ppm)	% Resistant $HgCl_2$	% Resistant $HgCl_2$	% Resistant PMA	[Hg] (ppm)
A2 - 3/29/72	6.5	--	<0.2 ± .02	3.1	7.0	—	0.27 ± .03	33.6	11.4	—	0.77 ± .04
B2 - 4/03/72	—	--	—	7.5	29.8	—	0.82 ± .02	22.5	31.0	—	0.68 ± .03
Rhode River 4/13/72	4.8	—	<0.2 ± .02	7.3	23.8	—	0.10 ± .01	5.9	—	—	—
B1 - 5/15/72	4.2	—	<0.2 ± .02	—	8.2	—	0.17 ± .01	—	10.9	—	—
A2 - 6/01/72	0.3	--	—	<0.4	1.5	—	0.37 ± .02	1.7	2.9	—	3.0 ± .90
B2 -10/05/72	13.2	--	0.09 ± .01	35.0	6.3	—	0.97 ± .04	35.7	—	—	—
EB1-10/06/72	—	—	—	—	4.8	—	0.04 ± .00	31.0	--	—	—
A2 -12/05/72	6.0	--	0.04 ± .01	8.1	1.5	—	0.20 ± .08	1.8	—	—	—
B2 - 1/04/73	8.9	22.4	—	—	3.3	5.5	0.67 ± .01	—	18.8	—	0.09 ± .01
B2 - 5/24/73	8.3	19.6	—	—	6.0	8.2	—	—	6.1	31.8	0.05 ± .00
EB1- 5/25/73	0.08	0.14	—	—	0.31	—	—	—	0.23	53.5	0.06 ± .05

[a] Dilutions of material were spread on basal medium, with and without 6 ppm of $HgCl_2$ or 0.3 ppm of PMA added. The percent of the total, viable, aerobic, heterotrophic bacterial population resistant to mercury was calculated.

[b] Water was filtered through sterile 8 um pore size Millipore filters.

[c] Supernatant solution resulting from centrifugation of sediment at 1,610 x G for 20 min.

[d] Collected with #20 nylon mesh plankton net.

[e] See Fig. 7, Section V.

The following experiments describe the resistance characteristics of pure cultures of bacteria grown in the presence of selected mercury compounds. Inoculum size, physiological age, and aeration of broth cultures were all found to be factors influencing mercury resistance. Hence, these parameters were carefully controlled in each experiment. Two hundred and forty-nine pure, freshly isolated, $HgCl_2$-resistant cultures were tested for resistance to a series of types and concentrations of mercury compounds by replicating from a master agar plate to a set of mercury-containing plates using sterile Velvetine cloth (Table 6). Groups of cultures which were strongly resistant to a selected compound showed a corresponding high resistance to other compounds of mercury. This suggests that the acquisition of resistance to a single compound of mercury confers a generalized resistance to mercury compounds. The comparative resistance of bacteria from water and sediments were established in an experiment with 131 cultures isolated from samples collected at a single station in Baltimore Harbor, Maryland (Fig. 2). In this particular sample, sediment populations were more resistant to different types and concentrations of mercury compounds. Similarly, the percent of total mercury-resistant bacteria was greater in sediment samples than in water samples. Differences in resistance can be explained by the fact that sediment and water bacterial population distributions are significantly different, but not always by intrinsic differences in mercury resistance, because the relationship between water and sediment in terms of percent resistance is not consistent (Section V).

Table 6. CROSS RESISTANCE OF BACTERIAL CULTURES TO MERCURY COMPOUNDS

	Percent of cultures resistant					
	Group[b]					
Test compound[a]	24 PMA	15 PMA	3 PMA	3 Me HgCl	100 $HgCl_2$	50 $HgCl_2$
24 ppm PMA	--	--	--	27.9	36.4	9.4
15 ppm PMA	--	--	--	90.8	63.6	20.8
3 ppm Me HgCl	85.8	89.6	6.1	--	100.0	29.2
100 ppm $HgCl_2$	28.6	24.1	0.0	25.6	--	--

[a]Each compound was incorporated into a solid growth medium.

[b]Each culture was classified according to the maximum amount of mercury tolerated (ppm).

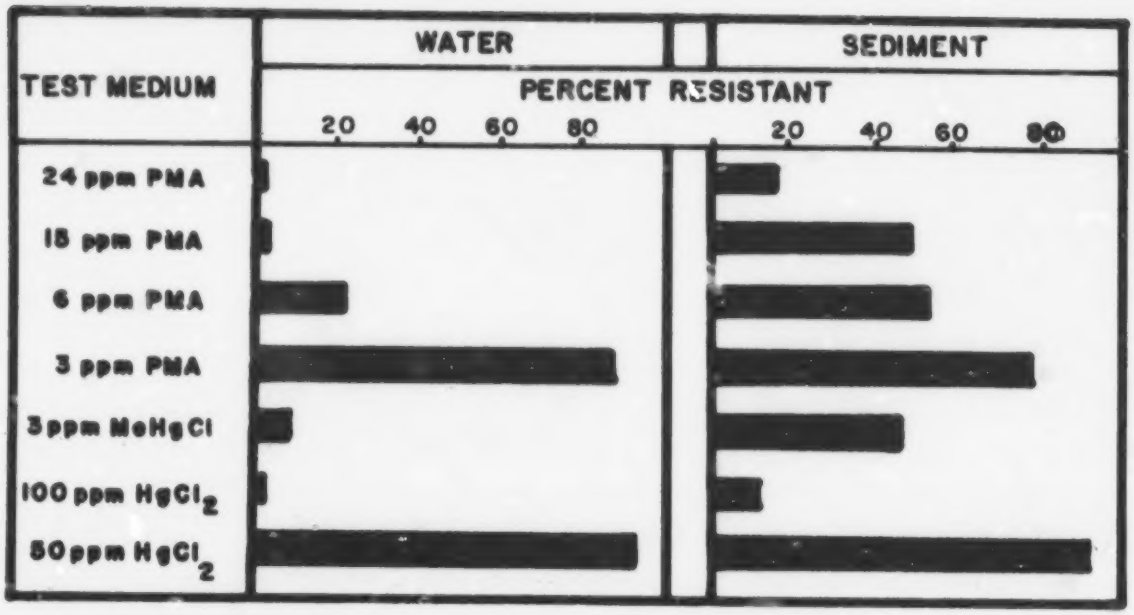

Figure 2. Comparative percentages of mercury-resistant isolates from sediment and water. (Reproduced with the permission of the Marine Technology Society.)

Mercury resistance in bacteria is often accompanied by resistance to other heavy metals and to drugs (5, 6, 7, 8). Resistance is believed to be conferred by factors borne by extra-chromosomal fragments ("plasmids") which are transferrable. A group of representative mercury-resistant bacteria were screened for resistance to a set of heavy metal ions using a broth medium. Variable patterns of resistance, particularly to Ag^{+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Cr^{+6}, and Hg^{2+} were observed (Table 7), suggesting that resistance was not generalized, i.e., that determinants of resistance are independent. The possibility of plasmid-mediated resistance among these bacteria is currently being investigated in our laboratory.

Bacterial Adaptation to Mercury Resistance

It was observed that, after serial-passages of bacteria through growth medium in the absence of mercury, resistance to mercury decreased significantly. When 30 cultures were grown in the absence and presence of inorganic and/or organic mercury, followed by replicate plating onto mercury-containing media of various types, it was found that growth of bacteria in the presence of mercury clearly decreased sensitivity to this metal (Table 8). "Adaptation" for growth in the presence of mercury compounds had been observed earlier by others (5, 9, 10). This phenomenon has been found to be a consequence of the "induction" of enzymes for the detoxification of mercury compounds via reductive decomposition of mercury compounds to volatile Hg^{o} (8, 11, 12). This

activity may be particularly relevant to the short term response of natural bacterial populations to mercury pollution, for the induction time frame may be in terms of only minutes or hours, under the proper conditions.

Table 7. HEAVY METAL RESISTANCE OF MERCURY-RESISTANT BACTERIA

Culture No.	Maximum concentration (ppm)[a] -- Metal ion[b]									
	Al^{3+}	Pb^{2+}	Ag^{+1}	As^{+5}	Co^{2+}	Cu^{2+}[c]	Zn^{2+}	Cd^{2+}	Cr^{+6}	Hg^{2+}
72	167.0[d]	167.0	16.7	501.0	334.0	334.0	55.1	100.2	668.0	4.0
119	167.0	167.0	16.7	501.0	55.1	334.0	167.0	167.0	1.7	20.0
187	16.7	167.0	1.7	501.0	55.1	334.0	110.2	50.1	668.0	16.0
132	16.7	167.0	1.7	501.0	167.0	334.0	167.0	167.0	16.7	40.0
85	167.0	167.0	1.7	501.0	167.0	334.0	55.1	50.1	100.2	12.0
639	16.7	167.0	1.7	501.0	110.2	334.0	55.1	100.2	50.1	12.0
94	16.7	167.0	1.7	501.0	55.1	334.0	55.1	16.7	334.0	24.0
244	16.7	167.0	1.7	501.0	55.1	334.0	55.1	50.1	100.2	50.0
127	16.7	167.0	1.7	501.0	18.4	334.0	18.4	16.7	16.7	24.0

[a] One drop of culture was added to 3 ml of basal broth containing dilutions of filter-sterilized solution of heavy metal, and the tubes were incubated, without agitation, at 25 C for 7 or more days. Tubes showing turbidity were scored positive. Uninoculated and inoculated controls with and without metals were included in the assay.

[b] Metal ion salts added were: $AlCl_3 \cdot 6H_2O$, $Pb(C_2H_3O_2)_2 \cdot 3H_2O$, $AgNO_3$, $Na_2AsO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $(3\ CdSO_4) \cdot 8H_2O$, $(NH_4)_2CrO_4$, and $HgCl_2$.

[c] Broth with salt added was adjusted to pH 7 with 1 N NaOH and filter-sterilized.

[d] Maximum concentrations tested were: Al - 167.0, Pb - 167.0, Ag - 167.0, As - 501.0, Co - 668.0, Cu - 334.0, Zn - 334.0, Cd - 334.0, Cr - 668.0, Hg - 100.0.

Table 8. ADAPTATION TO MERCURY RESISTANCE[a]

Test medium	Before growth in Hg		After growth in Hg	
	Medium grown in	% Cultures resistant	Medium grown in	% Cultures resistant
3 ppm Me HgCl	Control	20	100 ppm HgCl	63
24 ppm PMA	Control	3	100 ppm HgCl	77
24 ppm PMA	Control	7	50 ppm HgCl	77
24 ppm PMA	Control	7	3 ppm PMA	73

[a]Total number of cultures tested = 30.

Pseudomonas culture #5, when grown in the absence of $HgCl_2$, showed decreased resistance to $HgCl_2$. The same culture also evidenced a relatively decreased ability to volatilize radiolabeled Hg from a buffered suspension of cells containing ^{203}Hg-labeled $HgCl_2$ (Table 15, Section V). These results indicated the capability to metabolize inorganic Hg in this bacterial strain is inducible and related to mercury resistance. The volatile radioactive product of the reaction was isolated and characterized as Hg^0 (Section II), in agreement with the findings of others (9, 12). A simplified system, consisting of non-proliferating cells in a phosphate-buffered (pH 7.0), artificial, estuarine salts solution (PES), was used to establish kinetic parameters. When the suspension was bubbled with nitrogen, instead of air, Hg^0 formation was diminished by as much as 45%. This observation is possibly related to effect of anaerobiosis upon mercury resistance of mixed bacterial populations described above. The reaction was dependent upon cell concentration and exhibited saturation kinetics, characteristics which are attributable to enzyme-catalyzed reactions. At 25 C, the half saturation constant (Km) was 20 ppm of $HgCl_2$ and the maximum velocity of reduction was 35.4 μg/mg dry weight of cells/min.

Similarly, bacterial adaptation to an organic mercury compound, PMA, was investigated using Pseudomonas strain 244, which was originally isolated from PMA-containing medium. Figure 3 shows the change in resistance of the culture to a test concentration of 24 ppm of PMA, when grown with and without 6 ppm of PMA "inducer." In contrast to the non-induced culture, the induced culture, when plated on 24 ppm PMA medium, was able to grow and maintain resistance for a long period of time. The number of PMA resistant colony forming units (CFU) in the induced culture rose 200-fold, whereas CFU of the non-induced culture decreased 30-fold. In either case, these changes were preceded by a transient, but reproducible, increase in resistance prior to the onset of growth. This rapid response is consistent with the induction of an enzyme(s). The experiment was repeated using 6 ppm of HgCl as inducer. In contrast to the previous experiment,

resistance to 24 ppm of PMA rose to only a transient 19-fold increase. This difference may be the result of loss of Hg inducer, for it has been observed that Hg is chemically reduced and volatilized in the growth medium employed (Section VI).

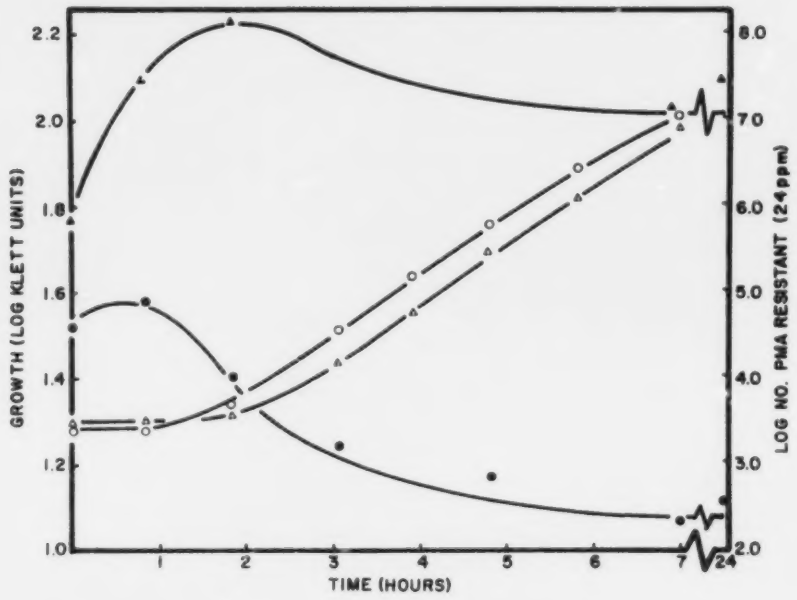

Figure 3. Induction of resistance to PMA by Pseudomonas 244. Broth with (triangles) and without (circles) 6 ppm of PMA was inoculated with cells. Growth was measured turbidimetrically (open symbols), and numbers of cells resistant to 24 ppm of PMA/ml were determined (closed symbols).

To ascertain the fate of PMA, the above experiment was repeated, using Pseudomonas 244 growing in a ^{203}Hg-labeled PMA medium (Fig. 4). Duplicate flasks, with and without inoculation, were assayed for cell-bound and total radioactivity at hourly intervals. During the period, in which it was shown in the previous experiment (Fig. 3), that resistance to PMA increased rapidly, a net loss of 39% and a cell accumulation of 4% of the label in the inoculated flask occurred. The uninoculated flask showed no significant decrease during the same period of time.

A group of representative bacteria was surveyed for the ability to volatilize Hg from labeled PMA-containing suspensions (Table 9). Analysis of the vapor phase of cultures on PMA agar by atomic absorption spectrophotometry and gas liquid chromatography (1) indicated that the cultures degraded PMA to Hg^{o} and benzene vapors (Fig. 5). The apparent periodicity of Hg evolution was an artifact of the sampling procedure. No organomercurials were detectable among the gaseous products. The

reductive decomposition of PMA, methyl mercury (Me Hg) and ethyl mercury has also been reported by others (2, 10, 13).

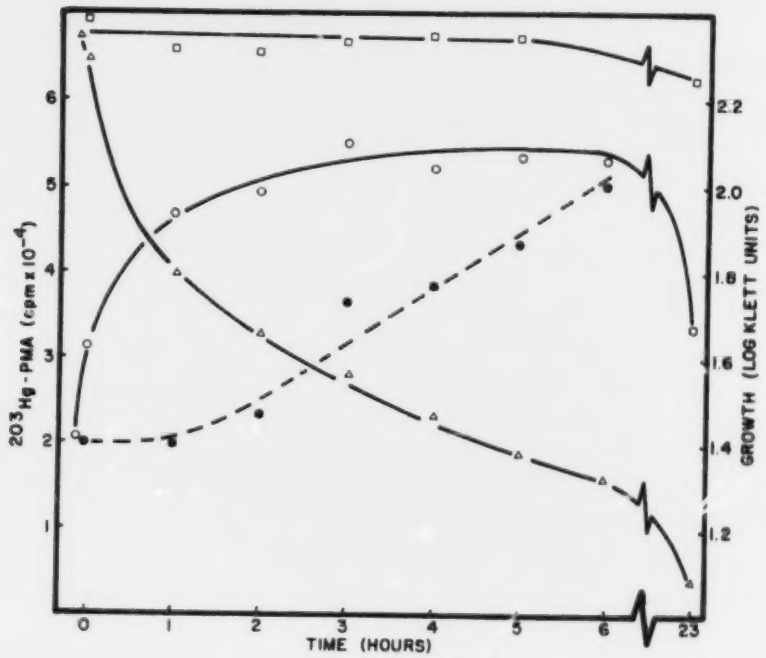

Figure 4. Uptake and metabolism of PMA by Pseudomonas 244. Cells were added to broth containing 6.08 ppm of radiolabeled PMA after 5 min. preincubation. Radioactivity in 0.1 ml of cell suspension (triangles) and in 1.0 ml of cell suspension collected on a membrane filter (open symbols) were determined. 0.1 ml samples of uninoculated broth (squares) were also analyzed. Growth was measured turbidimetrically (closed symbols).

Washed, non-proliferating cells of Pseudomonas 244 retained the ability to metabolize PMA for extended periods of time and were used to study the process in detail. Aerated suspensions of the cells containing ^{203}Hg-labeled PMA in PES were used to assay decomposition through the loss of radioactivity. Activity was dependent upon cell density and aeration driving off the $^{203}Hg^{o}$ formed. Cultures previously grown and harvested from media with and without 6 ppm PMA supplementation were assayed (Fig. 6). As in the case of the production of Hg^{o} and Hg^{2+}, the induced culture was much more active. Cells of both cultures accumulated label rapidly, but the non-induced, non-PMA-metabolizing culture accumulated label significantly more than the induced culture. Thus, for both Hg^{2+} and PMA, resistance and metabolism were related and inducible phenomena.

Table 9. METABOLISM OF PHENYLMERCURIC ACETATE BY MERCURY-RESISTANT BACTERIA

Isolate number	Generic identification	Percent radioactivity remaining[a]	
		1 hour[b]	4 days[c]
244[e]	Pseudomonas sp.	52.0	42.5[d]
187[e]	Pseudomonas sp.	94.8	87.1
94[e]	Pseudomonas sp.	88.3	73.7
127[e]	Pseudomonas sp.	82.6	59.9
72	Arthrobacter sp.	94.8	63.2
132	Citrobacter sp.	94.6	65.7
85	Enterobacter sp.	92.3	85.1
21	Vibrio sp.	98.2	87.5
119	Flavobacterium sp.	42.7	43.3

[a] 0.1 ml PMA in 95% ethanol, final concentration = 0.4 ug PMA (5.75×10^5 cpm/µg), was added to 0.9 ml of pH 7.0 PES buffer containing approximately equal quantities of cells. The suspensions were incubated at 25 C, and 100 ul samples were withdrawn.

[b] With aeration.

[c] Stationary.

[d] Isolate 244 was incubated 2 days.

[e] Isolates 244, 187, 94, and 127 were Pseudomonas sp. types I, II, III, and IV, respectively.

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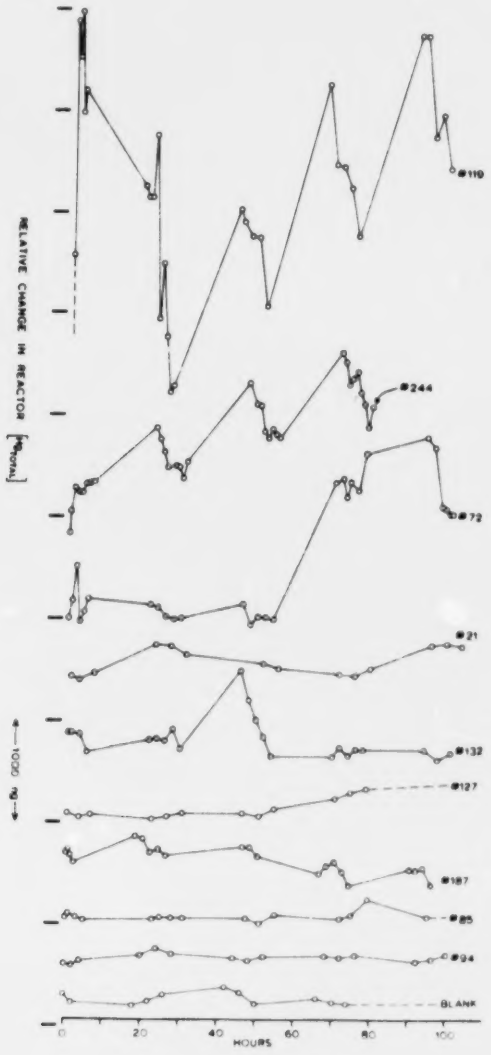

Figure 5. The change in mercury contained in the bioreactor atmosphere as a function of time is plotted for formation of Hg^0 from nine bacterial isolates from the Chesapeake Bay, each growing on six agar plates of basal media containing 0.3 ppm PMA. The small open circles represent $\pm$ std. dev. from the mercury calibration curves used. Hg values plotted here are not additively corrected for portions of reactor atmosphere removed at each sampling period. Note that in order to avoid overlapping, the individual curves are not referred to a common zero on the ordinate, but the 1,000 ng intervals shown on that scale provide an indication of the relative change in the amount of Hg present above each isolate referred to a control blank (uninoculated plates).

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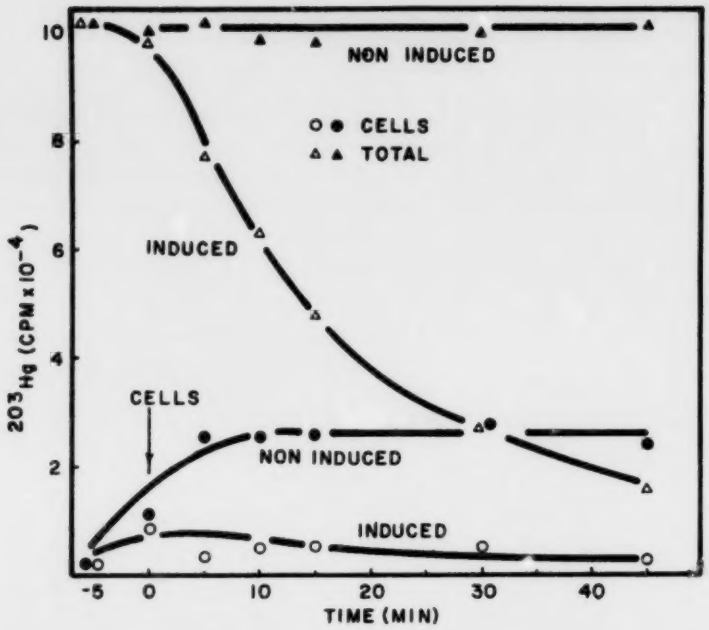

Figure 6. Uptake and metabolism of PMA by induced and non-induced resting cells of Pseudomonas 244. The reaction was begun when cells were added to the buffered PMA-salts mixture (arrow). 0.1/ml samples of cell suspension were taken at the indicated times.

The effect of salinity upon PMA metabolism by Pseudomonas strain 244 was tested by varying the total concentration of salts in the three salts solution, which is comprised of NaCl, $MgCl_2$, and KCl. Cells placed in three salts (33% sea water strength) buffered with 0.01 M tris (hydroxymethyl) aminomethane (TRIS)-hydrochloride (pH 7.0) were as active as those in a salts solution at 82% of the strength of sea water (Table 10). However, when the salt content was reduced to 8.2%, the cells were inactive. Inhibition was partially due to decreased salt concentration and to TRIS buffer, since the same concentration of salts in the absence of TRIS gave partial activity. When 0.01 M phosphate-buffered solutions of NaCl, $MgCl_2$, and KCl of equal ionic strength were tested, it was found that Mg^{2+} ion, alone, satisfied the ionic requirement for activity. In phosphate-buffered suspensions, a concentration of Mg^{2+} of from 5 - 10 mM was optimum for PMA degradation. A similar Mg^{2+} requirement for the cell-free reduction of Hg^{2+} to Hg^{o} by a mercury-resistant strain of Escherichia coli has also been reported (14). Magnesium ion concentration also effects resistance of the organism to PMA, as shown by the data given in Table 11. The optimum concentration for resistance was found to be in the range 1.2 - 5.6 mM Mg^{2+}. This observation further established the certainty of the relationship of PMA resistance and PMA

metabolism. The magnesium effect is particularly interesting in view of the estuarine locale from which the organism was isolated. The optimum magnesium concentration approximated the average in situ concentrations of magnesium. We have shown that metabolism of PMA by Pseudomonas strain 244 is dependent upon cell concentration and exhibits saturation kinetics. At 25 C, the pH optimum was between pH 6 and 7, the half saturation constant (Km) was 16.7 ppm PMA, and the maximum velocity of degradation was 10.6 ug/mg dry weight of cells/min.

Table 10. EFFECTS OF SALTS AND INHIBITORS ON PMA METABOLISM BY PSEUDOMONAS STRAIN 244

Experimental condition	PMA metabolized (percent of control[a])	
	Initial rate	Net/30 min
82.0% sea water, Tris buffer	99.3	—
8.2% sea water, Tris buffer	15.0	--
8.2% sea water, no buffer	56.1	--
Tris buffer, alone	5.6	--
NaCl, phosphate buffer[b]	26.1	--
KCl, phosphate buffer[b]	0.0	--
$MgCl_2$, phosphate buffer[b]	99.3	—
0.01 M KCN[c]	56.6	74.2
0.01 M NaN_3[c]	100.0	82.4
0.01 M $Na_2H\ AsO_4 \cdot 7H_2O$[c]	72.0	73.5

[a] 0.01 M buffered-"three salts" (pH 7.0) containing 6 ppm PMA and approximately 0.1 gm dry weight of cells/ml, aerated at 25 C.

[b] Concentration equivalent to total ionic strength of "three salts" solution.

[c] Inhibitors incubated with cells 5 min before reaction started in phosphate-buffered three salts.

Table 11. EFFECT OF MAGNESIUM ION CONCENTRATION ON PMA RESISTANCE IN PSEUDOMONAS STRAIN 244

Magnesium concentration (mM)	Growth (days)[a] PMA concentration (ppm) 0	10	15	20	25	30
0.1	+1	+3	--	--	--	--
1.2	+1	+1	+3	+3	+3	--
5.6	+1	+1	+1	+3	+3	+3
11.2	+1	+1	+1	+3	+3	+3
22.3	+3	+1	+1	+3	+3	+3

[a]0.01 ml of culture was transferred to tubes of basal broth containing varying concentrations of $MgCl_2$ and PMA. The tubes were incubated at 25 C, with shaking, and observed up to 7 days for growth.

Cellular Site of Mercury Binding and Metabolism

The effects of several inhibitors of oxidative phosphorylation were tested in Pseudomonas strain 244 PMA-metabolizing system to see if active transport of PMA into the cells was essential for metabolism (Table 10). Potassium cyanide (10 mM) caused considerable inhibition of the initial rate of reaction, but none of the inhibitors caused more than a 30% net decrease in the PMA metabolized after 30 min. The partial inhibitions may have resulted from the formation of complexes of phenylmercuric ions with the inhibitors. Cells treated with sodium arsenate or sodium azide bound considerably more label than did the control, but cyanide-treated cells bound less label. These results suggested that PMA metabolism takes place on, or near, the cell surface. A similar conclusion was reached by Tonomura et al. (10), who used a PMA-resistant Pseudomonas. The cellular site for binding of PMA was examined in the following way. Cells of Pseudomonas strain 244 were grown in the presence of ^{203}Hg-labeled PMA for 2/3 generation time, after which the cells were fractured by mechanical shear. PMA degradation was stopped immediately by the breakage, and the cells were separated into crude subcellular fractions by differential centrifugation (Table 12). Supernatant solutions were separated from pellets, after sequential centrifugation at 3,000 x G for 15 min, 35,000 x G for 15 min and 126,000 x G for 1 hr. Approximately 75% of the cell-bound radioactivity was present in particulate fractions sedimented by 3,000 and 35,000 x G forces. On the basis of these findings, it was concluded that the majority of the PMA binds to components of the cell envelope.

Table 12. DISTRIBUTION OF RADIOACTIVITY IN ^{203}Hg-PMA-LABELED CELLS OF PSEUDOMONAS STRAIN 244[a]

Cell fraction[b]	Total counts/min[c]	Percent of total	Percent radioactivity recovered in fractions
Cell free extract	1.59×10^5	100.0	—
3,000 x G pellet	2.28×10^4	14.3	21.0
35,000 x G pellet	5.85×10^4	37.4	53.7
126,000 x G pellet	8.06×10^3	5.1	7.3
126,000 x G supernatant	1.96×10^4	12.3	18.0

[a]5 ml of a 24 h culture growing in broth containing 6 ppm of PMA was added to 100 ml of fresh broth containing 6 ppm of PMA. The culture was incubated for 2 h, with shaking, at 20 C, and 20 microcuries of ^{203}Hg-labeled PMA were added. The culture was incubated an additional hour, and labeled cells were harvested by centrifugation.

[b]The labeled cells, suspended in chilled 0.01 M TRIS-buffered (pH 7.0) "three salts", were fragmented by two passages through a French Pressure Cell. The resulting cell-free extract was fractionated by differential centrifugation.

[c]Aliquots of each fraction were collected, and radioactivity was measured by liquid scintillation counting.

Cells of induced and non-induced cultures of Pseudomonas strain 244 were compared to identify a cell component responsible for mercury resistance. Preliminary investigations have shown no differences in gross lipid composition or in the morphology, by inspection of thin sections of cells using electron microscopy. However, striking cytological effects were observed in cultures which were less resistant to mercury and which were grown in the presence of $HgCl_2$ (Section VI). Cells of several cultures grown in the presence of $HgCl_2$ showed morphological similarities, suggesting that mercury impairs normal cell wall and membrane synthesis and function. This observation most likely is related to the propensity of mercury to bind to the cell envelope.

SUMMARY

This section described the sources of mercury-resistant bacteria and techniques used in this investigation. The cultural characteristics of mercury-resistant bacteria and the physiology of aerobic mercury metabolism were discussed.

Aerobic, heterotrophic bacteria resistant to mercuric ion were isolated from water, sediment, and plankton. They are predominately of the genus, Pseudomonas. Many of these organisms are also resistant to organomercurials, PMA (phenylmercuric acetate) and Me HgCl (methylmercuric chloride), deriving resistance from their ability to degrade mercury compounds with the formation of Hg^{o}. The capability of the organisms to produce Hg^{o} from Hg^{2+} and PMA is correlated with their ability to grow in the presence of compounds of mercury. Mercury resistance is also related to conditions of incubation, such as aerobiosis and temperature of incubation, as well as the salt content of the growth medium and the physiological age of the cultures.

SECTION V

MICROBIAL ECOLOGY OF MERCURY-RESISTANT BACTERIA IN CHESAPEAKE BAY

INTRODUCTION

Comparatively little is known about chemical and biological processes involved in the availability and mobility of inorganic mercury, the predominant pollutant form of mercury in the environment and a precursor of methyl mercury.

Microbial transformations of mercury, other than those involved in the formation of methyl mercury, have been described by several investigators. The reductive decomposition of organic and inorganic mercurials, with the formation of elemental mercury (Hg^0), have been reported for several genera of mercury resistant bacteria (1, 2, 8, 9, 13, 15, 16). This phenomenon may account for the low ambient levels of methyl mercury (17, 18) and, possibly, for the observed loss of Hg from contaminated sediments and soil (19, 20, 21, 22). However, the process of microbial Hg^0 generation should be emphasized as a potential mechanism for the mobilization of mercury and for the generation of substrate for methylation. The latter consideration is supported by evidence that Hg^0 is readily oxidized to Hg^{2+} *in situ* under the influence of dissolved oxygen and organic matter (23, 24).

Although direct pollution of the aquatic environment with mercury has been drastically curtailed during the early 70's, extensive sedimentary deposits exist which constitute a continuing methyl mercury hazard (25). In view of this prospect, an understanding of microbial processes in the generation of Hg^0 and Hg^{2+} is essential, particularly for evaluating environmental impact.

This report describes a study of the geographical and seasonal distributions of aerobic, heterotrophic, mercury-resistant bacteria involved in the formation of Hg^0 in Chesapeake Bay.

MATERIALS AND METHODS

Sampling

Water samples for bacterial plate counts were collected in sterile dilution bottles or sterile Niskin bag samplers (General Oceanics, Miami, Florida) 10 cm below the surface. Sediment samples for bacterial plate counts and mercury analysis were taken from the upper 5 - 10 cm layer using an Ekman dredge or Petite Ponar grab (Wildlife Supply Co., Saginaw, Michigan). Water samples for mercury analysis were collected in 0.5 N HNO_3-washed bottles and acidified promptly with 5 ml of concn. HNO_3 per liter. Horizontal plankton tows for 10 - 30 min, using plankton nets equipped with 20 mesh nylon cloth, provided ample material for analysis. The collected plankton were centrifuged, resuspended in sterile salts solution (see below) and either homogenized for bacterial plate counts or recentrifuged and lyophilized for mercury analysis. Appropriate controls and experiments eliminating the possibility that lyophilization affected the mercury analyses were made.

Mercury Analysis

Sediment samples were allowed to settle, the water layer, if any, was removed, and the sediments were air dried. Each sample of water or sediment was wet ashed according to Environmental Protection Agency procedures and analyzed by flameless atomic absorption spectrophotometry (26). Analyses were performed at the National Environmental Research Center, Cincinnati, Ohio and at the National Bureau of Standards, Washinton, D.C.

Isolation, Culture, and Identification of Bacteria

The medium employed for determination of the total viable count (TVC) and enumeration of mercury-resistant bacteria consisted of glucose, 2.0 gm; Casamino acids (Difco Laboratories, Detroit, Michigan), 5.0 gm; Yeast extract (Difco), 1.0 gm; and Bacto agar (Difco), 20 gm; per liter of artificial estuarine salts solution, i.e., 10.0 gm of NaCl, 2.3 gm of $MgCl_2 \cdot 6H_2O$, and 0.3 gm of KCl per liter. The salts mixture was adjusted to pH 7.2 and autoclaved at 121 C for 15 min. Mercury-containing media were prepared by adding freshly prepared solutions of $HgCl_2$ in sterile salts solution or PMA in 95% ethanol to sterile, molten agar medium. Suitable dilutions of material were prepared in sterile salts solution and spread on agar medium with and without added mercury within 5 hours of sampling. The inoculated plates were incubated for 7 days at 25 C, after which the colonies were counted. Colonies appearing on mercury-containing media were selected on a random basis and purified. The organisms were identified according to a taxonomic scheme originated by Shewan (27) which was modified by incorporation of the methods and procedures described by Colwell and Wiebe (28).

Assay for Mercury Evolution

Cultures were incubated with shaking in Erlenmeyer flasks of broth containing 6 ppm $HgCl_2$ for 16 h at 25 C and subcultured (20% inoculum size) in fresh medium, with subsequent incubation for 1 to 6 h. After incubation, the cells were centrifuged from the medium and washed twice in 0.01 M potassium phosphate-buffered (pH 7.0) estuarine salts (PES). After resuspension, the cells were divided into three portions: one was assayed directly, the second was autoclaved at 121 C for 15 min to serve as a control, and the third was centrifuged, resuspended in distilled water, and dried at 80 C for dry weight determination. Suspensions of cells in PES were added to PES in a test tube to 4.9 ml final volume. The suspensions were aerated with moist air to keep the cells evenly dispersed and to enhance the evolution of Hg^0 from solution. After 5 min aeration, 100 ul of ^{203}Hg-labeled $HgCl_2$ (Amersham Searle Corp., Arlington Heights, Illinois) was added. Samples (100 ul) were removed at intervals and mixed with 10.0 ml of 3a70 preblended liquid scintillation vials. Duplicate samples were filtered through 0.45 micron Millipore filters (Millipore Corp., Bedford, Massachusetts) and washed thrice with 1.0 ml volumes of PES at 25 C. The filters were immersed in 10.0 ml scintillation cocktail. Samples were counted in an Intertechnique model SL-40 liquid scintillation spectrophotometer (Teledyne Isotopes, Westwood, New Jersey). Radioactivity, expressed in disintegrations per min, was calculated automatically with a channels ratio program. Corrections for decay (47 day half life) were made when necessary.

Statistical Analyses

Data expressed as percent were converted by arc sine transformation (29) to approximate a normal distribution. Multiple stepwise linear regressions were performed by an IBM 1108 computer using biomedical program BMD03R from the University of Maryland Computer Center Library.

Isolation of Microbial Populations Directly from Water and Sediment

Bacteria and seston were recovered from water samples by centrifugation at 13,200 x g for 15 min. The greenish-brown material was resuspended in sterile PES before use. Bacteria were extracted from sediments by a modification of the methods of Balkwill and Casida (30) developed by those authors for soils. One hundred grams (wet weight) of sediment was homogenized with 200 ml of sterile 0.1% sodium pyrophosphate in salts solution (PES) in a Waring blender for two 30 sec. blending intervals. The mixture was centrifuged at 365 x g for 5 min and the supernatant solution was decanted and recentrifuged at 365 x g. The supernatant solution was again decanted and centrifuged at 13,200 x g for 10 min. The straw-colored supernatant solution was discarded, and the pellet was resuspended in sterile PES and centrifuged at 475 x g for 10 min. The dark brown pellet was discarded, and the greenish brown supernatant solution was retained for use.

RESULTS

Experimental Sampling Stations

Chesapeake Bay was traversed longitudinally during two cruises, one in May 1972 and the other in February 1973, to survey total mercury concentrations and mercury resistant populations of bacteria in sediments (see Fig. 7). The results (Table 13) showed that a range of mercury concentrations exists in Chesapeake Bay sediments, with highest levels present in the two most industrialized areas, Baltimore Harbor (stations 2 and 3, Fig. 7) and the Elizabeth River near Norfolk, Virginia (station 20, Fig. 7). Bacterial resistance to $HgCl_2$, expressed as the proportion of the total viable, heterotrophic, aerobic population capable of growth in a medium containing 6 ppm of $HgCl_2$, was also greatest at these two sites. The data strongly suggest a possible causal relationship between ambient mercury levels and numbers of mercury resistant bacteria.

A total of 6 stations at three locations in the upper Bay, shown in Fig. 7 (B-1, 2; A-1, 2; and EB-1, 2), were monitored at approximately 1.5 month intervals over a 17 month period, Fall 1971 through Spring 1973. Samples of surface water (10 cm depth) and the upper sediment layer were routinely analyzed for numbers of bacteria resistant to 6 ppm of $HgCl_2$ and 3 ppm of PMA and for total mercury concentration. The three stations encompassed a spectrum of environmental quality and ambient mercury concentrations from heavily industrialized Baltimore Harbor to relatively clean Eastern Bay, the site of important fin and shellfish fisheries. Differences in mercury concentrations in sediments among the three locations were significant at the 0.05 level and did not change appreciably during the 17 month monitoring period (15). Techniques of sufficient accuracy and precision for detection of mercury in water samples were developed somewhat later in the study. Levels of from 0.00 to 0.68 ppb were detected in the water from the three sites. However, there were insufficient data to draw conclusions as to the relationship of water to sediment mercury concentration or to location. Colgate Creek (station B-2), a subestuary in Baltimore Harbor, was found to contain the highest concentrations of mercury found in Chesapeake Bay during the course of this study. This site was subsequently found to be heavily contaminated with high levels of other heavy metals and oil pollutants as well (O. Villa and J. Marks, personal communication). The sources of mercury at this station have not been identified directly, but the indications are that point sources, such as a paint factory or a hospital sewage outfall, in the area may be implicated. The two sites of study which are located near Annapolis Harbor, A-1 and A-2 shown in Fig. 7, are subject to heavy commercial marine traffic.

Relationship of Mercury Resistance and Metabolism in Bacterial Isolates

From the onset of this investigation, the working hypothesis was that the percent of bacteria in the autochthonous microbial population which demonstrated resistance to mercury could be used as an index of potential microbial activity in the generation of Hg^{o} from inorganic or organic

sources of mercury. The hypothesized relationship between mercury resistance and metabolism was suggested by the published observations cited previously, and was substantiated by a series of experiments.

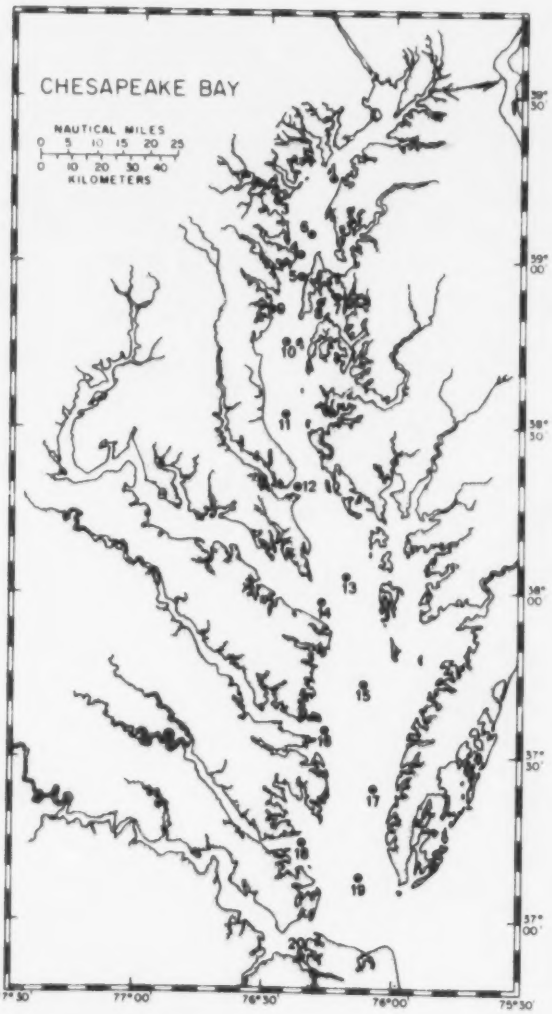

Figure 7. Experimental sampling sites in upper Chesapeake Bay. Station designations are: 2 = B-2 (Baltimore Harbor); 3 = B-1 (Baltimore Harbor); 4 = A-1 (Annapolis); 5 = A-2 (Annapolis); 7 = EB-1 (Eastern Bay); 8 = EB-2 (Eastern Bay); and 20 = Elizabeth River.

Table 13. TOTAL MERCURY CONCENTRATIONS AND PERCENTAGE OF THE TOTAL VIABLE BACTERIAL COUNT RESISTANT TO 6 ppm OF $HgCl_2$

Location[a]	Mercury concentration[b]		Percent resistant[c]	
	Sediment (ppm)	Water (ug/l)	Sediment	Water
1	0.220 ± 0.004			
2-Baltimore Harbor (B-2)	0.800 ± 0.070[d]	0.090 ± 0.010	22.8	28.5
3-Baltimore Harbor (B-1)	0.590 ± 0.620[e]	0.370 ± 0.010	8.2	4.2
4-Annapolis (A-1)	0.060 ± 0.040[e]	0.080 ± 0.010		
5-Annapolis (A-2)	0.200 ± 0.060[f]	0.490 ± 0.010	2.9	2.8
6	0.170 ± 0.010			
7-Eastern Bay (EB-1)	0.080 ± 0.030[d]	0.100 ± 0.020	1.3	0.5
8-Eastern Bay (EB-2)	0.040 ± 0.030[g]			
9	0.094 ± 0.002			
10	0.015 ± 0.007			
11	0.104 ± 0.013			
12	0.120 ± 0.010		2.6	0.2
13	0.073 ± 0.002			
14	0.100 ± 0.010		0.8	9.4
15	0.052 ± 0.011			
16	0.060 ± 0.020		1.8	0.7
17	0.023 ± 0.009			
18	0.070 ± 0.010		3.5	0.5
19	0.007 ± 0.002			
20 (May 1972)	0.860 ± 0.030		6.3	5.7
20 (February 1973)	0.280 ± 0.013	0.070 ± 0.010	2.2	2.3

[a] Numbers refer to sampling stations. Regularly sampled stations are underlined. Other stations were sampled in May 1972 and February 1973.

[b] Mean mercury concentration (dry weight) ± average deviation of one sample, except where designated otherwise. Water values based upon unfiltered samples taken October 1972.

[c] One standard deviation is approximately 10% of "Percent resistant." Stations 3, 12, 14, 16, 18, and 20 assayed on May 1972 cruise and stations 2, 5, 7, and 20 assayed on February 1973 cruise.

[d] Ninety-five percent confidence interval of the mean mercury concentration of 8 samples.

[e] Ninety-five percent confidence interval of the mean mercury concentration of 6 samples.

[f] Ninety-five percent confidence interval of the mean mercury concentration of 9 samples.

[g] Ninety-five percent confidence interval of the mean mercury concentration of 4 samples.

Figure 8 dramatically illustrates the generation of Hg^{o} by a mixture of bacterial colonies growing on an $HgCl_2$-containing nutrient agar. Fogging of areas on an X-ray film exposed to the inoculated mercury-containing agar plates resulted from reduction of the Ag^{+} emulsion by Hg^{o}. Metabolism of $HgCl_2$ by individual mercury-resistant colonies, randomly selected from platings of sediment and water, was assayed by measuring the disappearance of ^{203}Hg from aerated, $HgCl_2$-containing, buffered salts suspensions of bacteria (Table 14). A large proportion of the isolates (77%) evolved ^{203}Hg in amounts which exceeded killed and uninoculated controls.

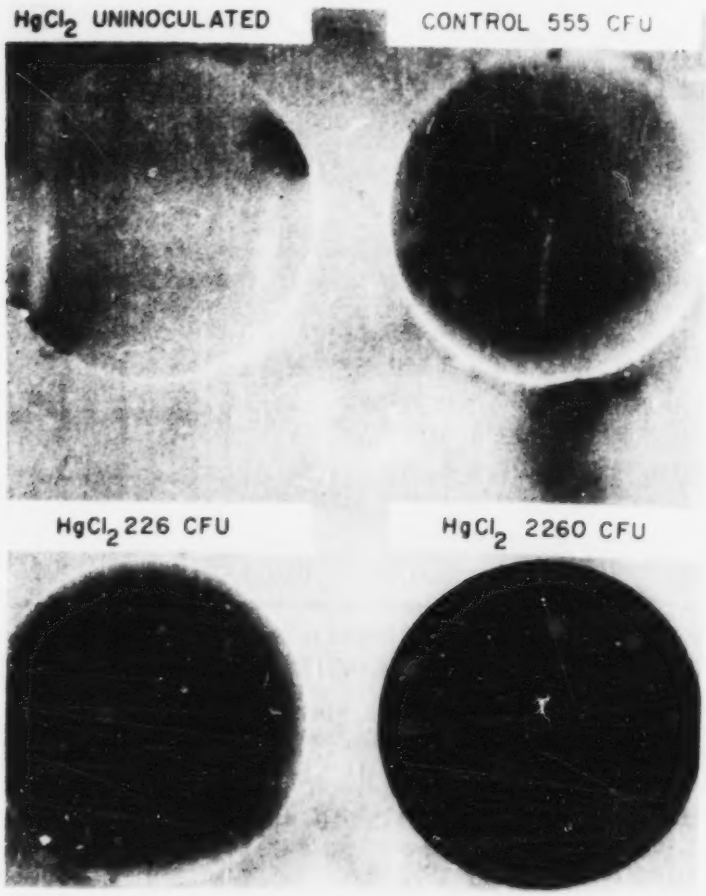

Figure 8. Elemental mercury evolution by bacteria grown on agar plates containing $HgCl_2$. Plates of nutrient medium, with and without 6 ppm of $HgCl_2$, were spread with dilutions of Colgate Creek (Station B-2) sediment. The plates were incubated, uncovered, under Kodax No-Screen X-ray film at 25 C for 1 week, after which the film was developed. "CFU" = colony forming units.

Table 14. $HgCl_2$ METABOLIZING BACTERIA

Source[b]	Culture	mg Cells (dry weight)	Percent change in (^{203}Hg)[a] Decrease: Live	Killed	Uninoculated	Cellular[c] uptake	Incubation time (h)
B-2	1	0.99	12.6	5.4	4.6	22.7	48
B-2	3	2.36	38.1	8.5	4.5	51.8	72
B-2	4	1.17	1.1	5.5	4.6	29.6	48
B-2	5	1.08	83.4	3.2	—	4.8	1
B-2	7	0.84	5.6	4.1	8.0	26.6	24
B-2	8	1.17	15.0	5.9	4.6	21.0	48
B-2	10	0.42	26.1	1.1	4.5	31.8	72
B-2	11	0.84	33.4	7.6	—	20.0	1
B-2	12	1.02	9.0	2.5	4.6	20.6	48
B-2	13	0.17	1.7	1.2	4.5	8.8	72
B-2	14	1.26	21.9	7.3	4.6	36.5	48
B-2	15	0.72	14.7	2.7	—	23.8	1
B-2	16	1.41	81.6	6.5	—	16.0	1
B-2	17	0.72	11.8	—	8.0	—	24
B-2	19	0.81	31.4	3.0	4.5	30.2	72
B-2	20	0.51	30.4	7.5	4.5	20.2	72
EB-1	21	0.84	14.2	2.0	4.5	13.5	72
EB-1	24	0.84	33.3	3.2	4.5	11.8	72
EB-1	26	1.48	21.0	5.9	—	38.2	1
EB-1	28	1.04	37.8	4.8	4.5	30.0	72

[a] A suspension of washed cells, maintained at 25 C in 4.9 ml of PES and aerated via a capillary tube, was incubated for 5 min prior to the addition of 0.1 ml of ^{203}Hg-labeled $HgCl_2$ (final concentration of 6 ppm $HgCl_2$; approx. 100,000 CPM/ml). 0.1 ml samples were removed at zero time and at regular intervals to 10.0 ml scintillation cocktail for counting. Percentage decrease or uptake was based upon counts corrected for quenching and decay relative to radioactivity in the killed control at t = 0.

[b] Selected from 6 ppm $HgCl_2$ count plates of water and sediment from Colgate Creek and sediment from Eastern Bay. Of the 30 original colonies subcultured from the count plates to broth containing 6 ppm $HgCl_2$, 22 grew, with 17 actively metabolising Hg^{2+}.

[c] 0.1 ml aliquots were filtered through .45 μ Millipore filters and washed three times with 1.0 ml volumes of PES (25 C).

An examination of one of the most active isolates (culture #5) further suggested a correlation of resistance and metabolism. The organism was grown in the absence of $HgCl_2$, with concomitant diminution of its tolerance for, and ability to metabolize, $HgCl_2$ (Table 15). Experiments utilizing a closed system with a $HgBr_2$-KBr mercury trap (18), in which the radioactive volatile product of the reaction was quantitatively collected, suggested that Hg^o evolution accounted for the loss of Hg from the suspension (Table 16). Radioactivity in the acidified trapping solution was not benzene-extractable, nor were methyl or other alkyl mercury compounds detected in thin layer chromatograms of the dithizone derivative.

Table 15. MERCURY RESISTANCE AND METABOLISM OF BACTERIAL STRAIN NO. 5 GROWN IN THE PRESENCE AND ABSENCE OF $HgCl_2$

A. Resistance

Culture[b]	Growth[a]					
	$HgCl_2$ concentration (ppm)					
	4	8	12	16	20	24
Grown with $HgCl_2$	+	+	+	+	+	-
Grown without $HgCl_2$	+	+	+	-	-	-

[a] Tubes were inoculated with 1 drop of culture, incubated, with shaking, at 25 C, and observed daily for up to 21 days.

[b] Cultures were serially transferred twice with or without 6 ppm $HgCl_2$ in the growth medium. A 24 hour old culture was used to inoculate the test medium.

B. Metabolism

Culture[b]	mg Dry weight	Percent change in (^{203}Hg)/30 min[a]	
		Decrease in suspension	Uptake by cells
Grown with $HgCl_2$	0.39	38.8	13.7
Grown without $HgCl_2$	0.43	5.1	15.6

[a] A suspension of washed cells, maintained at 25 C in 4.9 ml of PES and aerated via a capillary tube, was incubated for 5 min prior to the addition of 0.1 ml of $HgCl_2$ (final concentration of 6 ppm $HgCl_2$; approx. 300,000 CPM/ml). 0.1 ml samples were removed at zero time and at regular intervals to 10.0 of scintillation cocktail. Percentage decrease in the suspension or increase in the cellular (non-filterable) radioactivity was based upon counts corrected for quenching.

[b] The cultures were grown for 2 h at 25 C, with shaking, in broth in the presence and absence of 6 ppm $HgCl_2$. Inocula were cells grown for 24 h with and without 6 ppm of $HgCl_2$ in the growth medium. Equal turbidities were attained in each culture at the end of the incubation period.

Table 16. CHARACTERIZATION OF THE VOLATILE Hg METABOLITE PRODUCED BY PSEUDOMONAS STRAIN 5

Experiment	Sample	Percentage of added (^{203}Hg)[a]
I[a]	Suspension after 60 min	0.67
	Cells after 60 min	0.98
	$HgBr_2$-KBr trap after 60 min	85.00
	Benzene extract of trap	0.00
II[c]	a. Suspension after 90 min	0.80
	b. Cells after 90 min	0.60
	c. $HgBr_2$-KBr trap after 90 min	96.80
	d. First dithizone extract of trap	22.80
	e. Second dithizone extract of trap	76.20
III[d]	a. Dithizone extract of II c.	44.00
	b. Thin layer chromatography of III a. Hg^{2+} spot plus origin	44.00

[a] A flask containing 9.0 ml of PES containing ^{203}Hg-labeled $HgCl_2$ was connected via plastic tubing to a second flask containing 10.0 ml of $HgBr_2$-KBr trapping solution. The experiment set-up was such that the atmosphere was continually recirculated by means of a peristaltic pump as the flasks were shaken. After 15 min incubation, 1.0 ml of washed cells in PES was added to the first flask and incubation was continued. The trapping solution was examined for radioactivity before and after addition of cells to the system. Transfer of radioactive Hg to the trapping flask was insignificant in the absence of the bacterial suspension. At the end of the incubation, cells were filtered through a 0.45 micron Millipore filter and washed thrice with 1.0 ml volumes of PES.

[b] 4.1 ml dry weight of cells with 1.8 ppm $^{203}HgCl_2$ (131,000 dpm/ml). Five volumes of trapping solution were acidified with concentrated HCl to a concentration of 1 N, and were extracted with 1 volume of benzene.

[c] 1.9 mg dry weight of cells with 1.5 ppm $^{203}HgCl_2$ (202,000 dpm/ml). One volume of trapping solution was extracted with 2 volumes of a 1% solution of dithizone in benzene. The extraction was repeated.

[d] One volume of trapping solution (Expt. II) was extracted with 2 volumes of 0.8% dithizone in benzene. The dithizonate derivative was spotted on a silica gel G plate (250 μ thickness) and developed with petroleum ether:diethyl ether (70:30) solvent. Spots were scraped off and collected in scintillation vials and suspended in an Aquasol (New England Nuclear) gel for counting. Dithizone extraction and chromatography were according to the methods of Westoo (31).

The microbial populations of water and sediment, collected by differential centrifugation, also carried out the reductive process. Recoveries of bacteria isolated by this method were essentially quantitative for water and approximately 1% for sediments, based upon total, viable, aerobic, heterotrophic bacterial counts. Assays for total and non-filterable radioactive mercury in suspensions containing live and sterile inocula and uninoculated controls were performed as described for the previous pure culture experiments (Table 17). Under the conditions of the assay, the microorganisms slowly released Hg^0. The rate of loss measured for the sterilized controls indicated some chemical reduction, i.e., non-biological reduction also occurred. The major difference between the Colgate Creek and Eastern Bay samples (B-2 and EB-1), respectively) was, in each case, the amount of mercury bound to non-filterable material. Lowering the initial Hg^{2+} concentration by an order of magnitude decreased the loss of Hg^0, but increased binding.

Analysis of Seasonal Data

Figures 9 and 10 illustrate changes in proportions of $HgCl_2$-resistant bacteria in water and sediment during the period from January 1972 through May 1973. A distinct periodicity was evident for both water and sediment, with a major peak in mercury-resistant bacteria observed in the Spring months of both 1972 and 1973 and a possible secondary peak in the Fall of 1972. Figures 9 and 10 also show the relative differences in resistance among three of the stations. Several physical parameters were found to demonstrate a seasonal periodicity (Fig. 11) characteristic of the Chesapeake Bay estuary (32).

The data were subjected to multiple stepwise regression analysis to detect possible relationships between the selected physical parameters and number of resistant organisms and to establish a basis for comparison of the three sites. The percentages of bacteria resistant to $HgCl_2$ in water and sediment were treated as dependent variables, respectively. Independent variables, whose partial regression coefficients were significantly greater than zero (t test of significance), are presented in Table 18. Standard partial regression coefficients were also calculated to show the relative contribution of each variable to the regression equations (29). When each site was considered separately, it was evident that there were differences in factors affecting mercury resistance among the sites (sample 1, 2 and 3). When data for all six stations were added to the regression equation, the individual measured parameters did not adequately account for changes in resistance (sample 4). Three stations were selected on the basis of similarities in sediment type and differences in sediment concentrations (sample 5). Percentage of resistance in water and sediment for each site were observed to be related, but differences between water and sediment were not significant (0.05 significance level). However, average differences in percentage of resistance in water and sediment, respectively, among the three locations were significant (0.05 significance level). Resistance in sediments was found to be correlated positively with water transparency (Secchi disc), dissolved oxygen, and total mercury concentration in the sediment. Resistance was not observed to be related to total viable count.

Table 17. Hg^0 EVOLUTION BY NATURAL POPULATIONS OF BACTERIA ISOLATED FROM WATER AND SEDIMENT

Sample	$[HgCl_2]$ (ppm)	TVC per ml	Time (h)	Percent change in (^{203}Hg)[a] Decrease: Uninoculated	Live suspension	Sterile suspension	Cellular uptake
B-2 sediment	6.0	1.2×10^5	1		6.6	1.7[b]	38.3
B-2 sediment	6.0	1.2×10^5	2		10.3	0.0	38.7
B-2 sediment	6.0	1.2×10^5	3		11.0	3.0	40.2
B-2 sediment	6.0	1.2×10^5	24		11.6	6.3	46.4
B-2 sediment	6.0	1.2×10^5	48		13.7	9.9	50.5
EB-1 sediment	6.0	1.0×10^5	1		7.4	1.7[b]	9.4
EB-1 sediment	6.0	1.0×10^5	2		10.5	3.9	10.9
EB-1 sediment	6.0	1.0×10^5	3		8.8	2.2	11.7
EB-1 sediment	6.0	1.0×10^5	24		9.1	9.1	15.6
EB-1 sediment	6.0	1.0×10^5	48		11.8	10.7	16.8
B-2 water	6.0	7.0×10^5	1		7.7	1.3[b]	6.8
B-2 water	6.0	7.0×10^5	2		9.3	1.0	7.2
B-2 water	6.0	7.0×10^5	3		11.1	0.1	9.0
B-2 water	6.0	7.0×10^5	24		15.6	0.7	9.7
B-2 water	6.0	7.0×10^5	48		20.6	2.4	10.7
B-2 water	6.0	1.8×10^6	2	5.3	5.8	4.6[c]	5.8
B-2 water	6.0	1.8×10^6	18	8.0	10.5	7.8	6.0
B-2 water	6.0	1.8×10^6	42	4.6	10.2	7.5	7.7
B-2 water	6.0	1.8×10^6	66	4.5	12.7	7.2	9.6
B-2 sediment	0.6	4.6×10^5	1		0.3	1.4[b]	95.0
B-2 sediment	0.6	4.6×10^5	2		1.8	4.1	101.0
B-2 sediment	0.6	4.6×10^5	3		1.8	5.5	96.5
B-2 sediment	0.6	4.6×10^5	24		4.1	7.1	99.9
EB sediment	0.6	2.5×10^6	1		4.0	4.7	40.0
EB sediment	0.6	2.5×10^6	2		4.2	5.7	44.3
EB sediment	0.6	2.5×10^6	3		5.5	7.7	44.7
EB sediment	0.6	2.5×10^6	24		7.7	13.6	57.2

[a] 0.1 ml of $^{203}HgCl_2$ in PES was added to a 4.9 ml PES suspension of seston or sediment extract (prepared as described in Materials and Methods). The tubes of suspension were aerated, via a capillary tube, with moistened air for 15 min prior to each sampling. Radioactivity in 0.1 ml samples of suspension and non-filterable material was measured and corrections for quenching and decay were made.

[b] Sterilized by heating at 121 C for 15 min. No attempt was made to maintain sterility after the start of the incubation.

[c] Sterilized with chloroform. No attempt was made to maintain sterility after the start of the experiment.

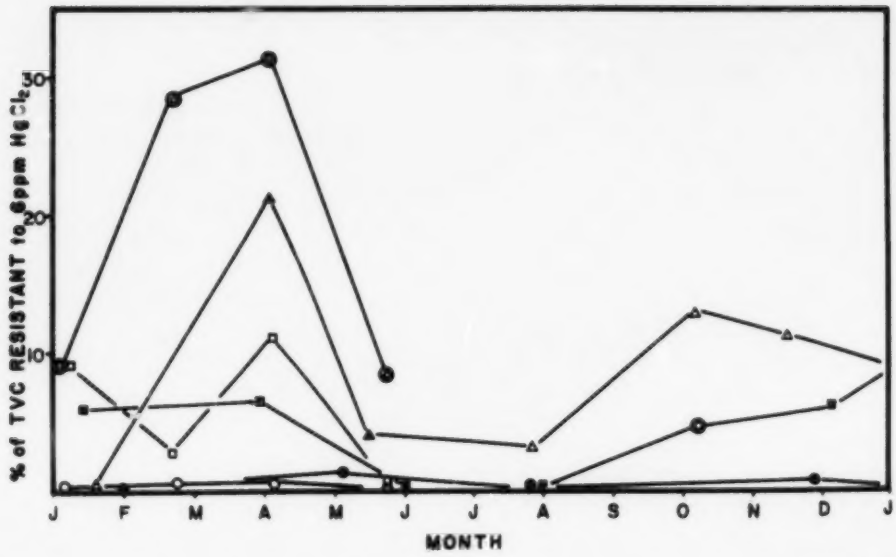

Figure 9. Seasonal variation in percent of $HgCl_2$ resistant bacteria in water. The percent of total viable, aerobic, heterotrophic bacterial count (TVC) capable of growth on a solid medium containing 6 ppm of $HgCl_2$ was determined. Water samples from Station B-1, 1972 (▲); Station B-2, 1972 and 1973 (△,◬); Station A-1, 1972 (◙); Station A-2, 1972 and 1973 (■,□); and Station EB-1, 1972 and 1973 (●,○), were plated and incubated at 25 C for 1 week.

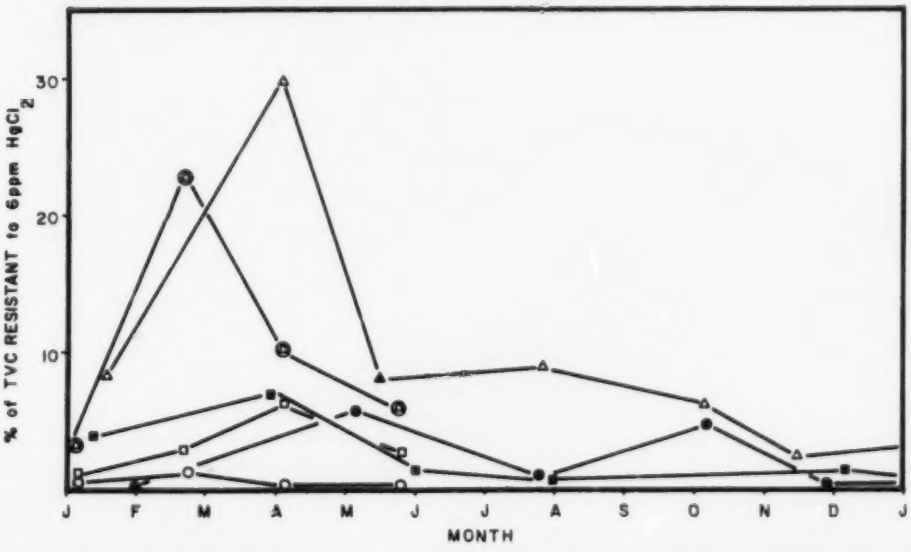

Figure 10. Seasonal variation in percent of $HgCl_2$ resistant bacteria in sediment. The percent of total, viable, aerobic heterotrophic bacterial count (TVC) capable of growth on a solid medium containing 6 ppm of $HgCl_2$ was determined. Sediment samples from Station B-1, 1972 (▲); Station B-2, 1972 and 1973 (△,◬); Station A-2, 1972 and 1973 (■,□); and Station EB-1, 1972 and 1973 (●,○) were plated and incubated at 25 C for 1 week.

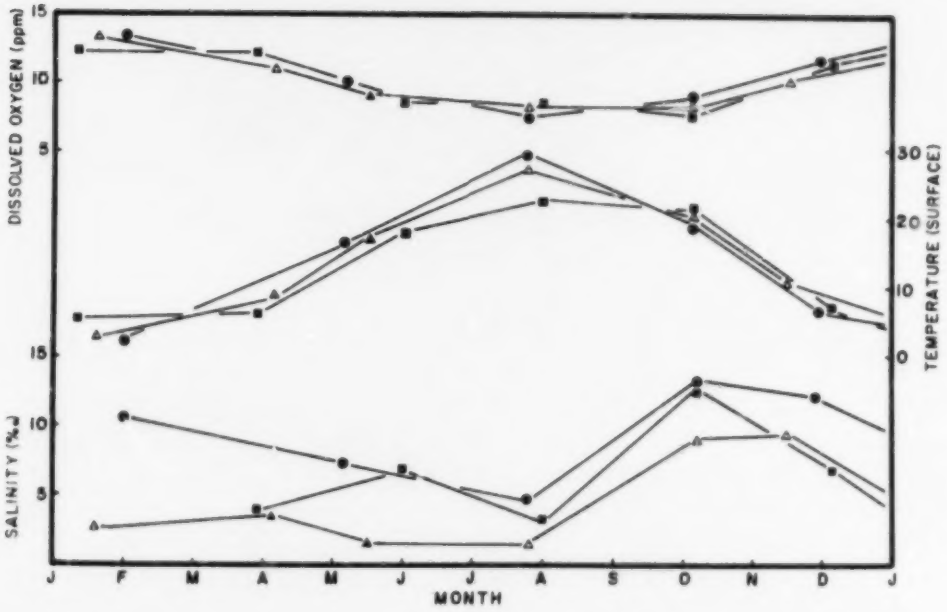

Figure 11. Seasonal variation in salinity, temperature, and dissolved oxygen concentration in surface waters from three locations in Chesapeake Bay. Station B-1 (▲); Station B-2 (Δ); Station A-2 (■); and Station EB-1 (●).

Analysis of plankton indicated wide fluctuations in mercury levels, but no significant differences (0.05 significance level) in average concentrations among the three areas. The relationship between mercury concentration in plankton and numbers of mercury-resistant bacteria in water or sediment was not found to be significant. Limited data, however, indicated possible seasonal parallels between the sizes of the Hg^{2+} resistant bacterial microflora of the plankton and the water and sediment.

Because phenylmercurials are now the only organomercurials registered for industrial use (T. Adamczyk, Registration Division, Environmental Protection Agency, personal communication), phenylmercuric acetate (PMA) was also used as a test compound in these studies. Three ppm of PMA (1.79 ppm Hg) proved to be much more toxic than 6 ppm of $HgCl_2$ (4.43 ppm Hg) when incorporated in the growth medium. In fact, poor precision in viable counts were obtained because of few colonies and poor survival on the PMA medium. Nevertheless, the data suggest that bacterial resistance to PMA in water and sediment exhibit a periodicity paralleling that of Hg^{2+} resistance.

Table 18. MULTIPLE REGRESSION ANALYSIS OF SEASONAL DATA

Y	X_n[a]	Correlation coefficients Simple	Partial	Multiple	Partial standard regression coefficient	Significance level	df	Sample
% water	3	+0.08557	-0.90433	0.9871[b]	-9.14	0.05	10	1-A1, A2
% water	5	-0.76012	-0.84457	0.9871	-1.29	0.05	10	"
% sediment	6	+0.50568	+0.82607	0.9232	+0.42	0.05	10	"
% water	7	+0.79797	+0.81540	0.9640	+0.85	0.05	11	2-B1, B2
% water	9	-0.42607	-0.72052	0.9640	-0.57	0.10	11	"
% water	2	+0.79752	+0.94433	0.9731	+1.96	0.05	9	3-EB1, EB2
% water	4	+0.26347	-0.89642	0.9731	-0.95	0.10	9	"
% sediment	1	+0.79752	+0.94434	0.9950	+0.45	0.05	9	"
% sediment	3	+0.68272	+0.94348	0.9950	+0.64	0.05	9	"
% sediment	4	+0.45798	+0.95714	0.9950	+0.49	0.01	9	"
% sediment	5	+0.01878	-0.96038	0.9950	-8.30	0.01	9	"
% sediment	6	-0.14795	-0.92919	0.9950	-1.09	0.05	9	"
% sediment	8	-0.00870	-0.96131	0.9950	-7.30	0.01	9	"
% sediment	9	-0.15035	+0.94953	0.9950	0.67	0.05	9	"
% water	3	+0.67893	+0.60978	0.7358[c]	0.65	0.001	32	4-A1, A2 B1, B2 EB1, EB2
% water	2	+0.66167	+0.5777	0.8171	0.71	0.05	17	5-A2, B2 EB1
% sediment	1	+0.66167	+0.5777	0.8834[d]	0.47	0.05	17	"
% sediment	7	-0.47979	+0.50305	0.8834	0.59	0.10	17	"
% sediment	8	+0.14737	+0.53308	0.8834	1.55	0.10	17	"
% sediment	9	+0.66394	+0.59526	0.8834	0.83	0.05	17	"

[a] X_1 = % water; X_2 = % sediment; X_3 = TVC water; X_4 = TVC sediment; X_5 = surface water temperature; X_6 = salinity; X_7 = turbidity; X_8 = dissolved oxygen; X_9 = total mercury concentration in sediment. Only those variables whose partial regression coefficients were significantly >0 are included (see "Significance level" column).

[b, c, d] F value for analysis of variance for multiple linear regression is significant (0.10, 0.01, and 0.05 levels of significance, respectively).

Distribution of Types of Mercury-Resistant Bacteria

Representative colonies from platings of water and sediment on $HgCl_2$- and PMA-containing media were selected for further study. Cultures were purified and identified to genus for each of the individual samples examined during 1972 (Table 19). The majority of $HgCl_2$-resistant bacteria were cytochrome oxidase positive, non-pigmented, Gram-negative, asporogenous rods which produced H_2S, but did not utilise glucose or require added sodium ion for growth. PMA-resistant bacteria differed primarily in the utilization of glucose and production of diffusible pigments. Upon comparison of paired $HgCl_2$-resistant and randomly selected isolates from medium without Hg, it was found that differences in salt requirements and H_2S production were not significant (0.05 level of significance).

Preliminary results, however, indicated that there were detectable differences in the population distributions of the mercury-resistant and the total viable, aerobic, heterotrophic bacterial populations (34). The total population is now being more fully characterized to verify this observation. A total of 539 pure cultures isolated on 6 ppm $HgCl_2$-containing medium, collected in the period from October 1971 through December 1972, were characterized. On the basis of our modification of the scheme proposed by Shewan et al. (27), the cultures were placed into one of seven generic groups or an eighth category (less than 4% in the majority of cases) into which were lumped yeasts, molds, and unidentified bacteria. Chi-square analysis of the data (0.01 level of significance) indicated that there were significant differences in distribution spatially (Fig. 12), seasonally (Fig. 13), and between water and sediment (Fig. 14). The apparent diversity of genera was greatest at the Eastern Bay stations. The most conspicuous seasonal changes in population structure were in the Pseudomonas and Bacillus spp. (Fig. 12). Minima in percentage of the former occurred in the spring and fall, while a maximum of the latter occurred during the fall destratification. Water and sediment differed primarily in the proportions of Pseudomonas and Gram positive rods (Bacillus spp. plus coryneform bacteria). The genus Pseudomonas accounted for 66% of all $HgCl_2$-resistant bacteria in water and sediment. A much higher proportion, 93%, of the 70 PMA-resistant isolates were Pseudomonas spp., the majority of which were type II (27).

DISCUSSION

Experimental Sampling Stations

The range of mercury concentrations found in sediments of the regularly sampled stations appears to be related to the nature of the sediments and their locations (Table 13). The highest concentrations were found in the fine silty-clay sediments located near industrial activity or industrial plant outfalls. There was no significant difference in the mercury level of the sandy sediments of Station A-1 and the silty-clay sediments of Eastern Bay. The difference in mercury concentrations

Table 19. PHYSIOLOGICAL CHARACTERISTICS OF $HgCl_2$- AND PMA-RESISTANT BACTERIA

	Cytochrome[a] oxidase	Gram-negative	Green Soluble Pigment Produced	H_2S[b] produced	Na^+[c] required	Bacto OF medium plus 1% glucose[d] (aerobic/anaerobic)			
						Acid/—	Acid/Acid	Alkaline/—	—/—
A. $HgCl_2$ (6 ppm) resistant[e]									
No. of cultures	288	353	55	233	32	61	35	217	103
Percent	68.0	83.5	13.0	55.0	7.6	14.4	8.3	51.3	24.3
B. PMA (3 ppm) resistant[f]									
No. of cultures	64	69	15	40	6	38	2	28	0
Percent	92.8	100.0	21.8	58.0	8.7	55.0	2.9	40.6	0

[a]Method of Gaby and Hadley (33).

[b]From sodium thiosulfate or L-cysteine.

[c]Requirement for 1% NaCl for growth.

[d]Anaerobic tubes were layered with sterile mineral oil. Dash indicates no change in pH.

[e]Total of 423 isolates tested.

[f]Total of 69 isolates tested.

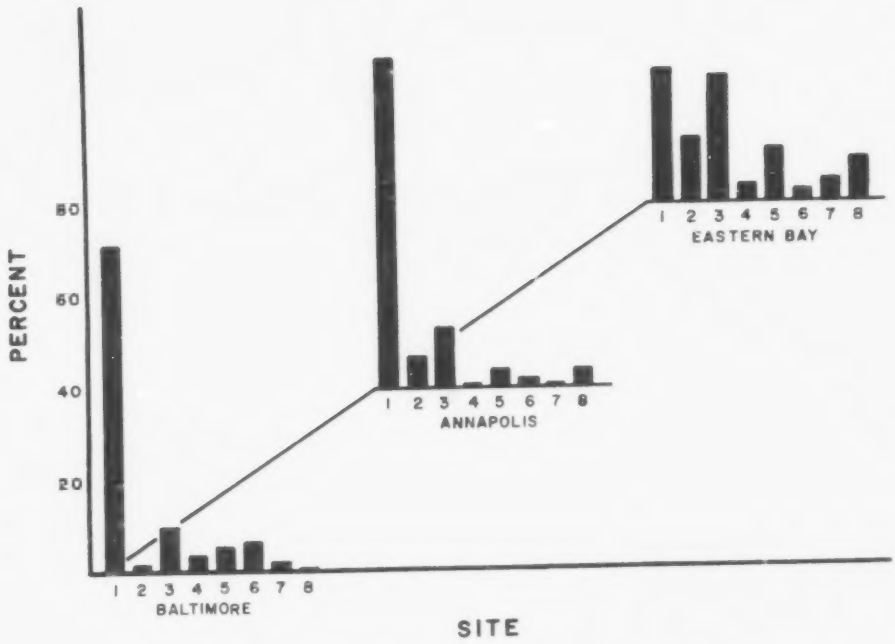

Figure 12. Distribution of $HgCl_2$ resistant, aerobic, heterotrophic bacteria isolated from water and sediment of Chesapeake Bay. Four hundred and eighteen pure cultures found to be resistant to 6 ppm of $HgCl_2$ were isolated between January 1972 and December 1972. These cultures were classified into 8 genera or generic groups: 1 = Pseudomonas, 2 = Vibrio and Aeromonas, 3 = Bacillus, 4 = coryneform, 5 = Cytophaga and Flavobacterium, 6 = Achromobacter, Alcaligenes, and Acinetobacter, 7 = Enterobacteriaceae, and 8 - unidentified. The cultures were isolated from B-1 and B-2 ("Baltimore"), A-1 and A-2 ("Annapolis") and EB-1 and EB-2 ("Eastern Bay").

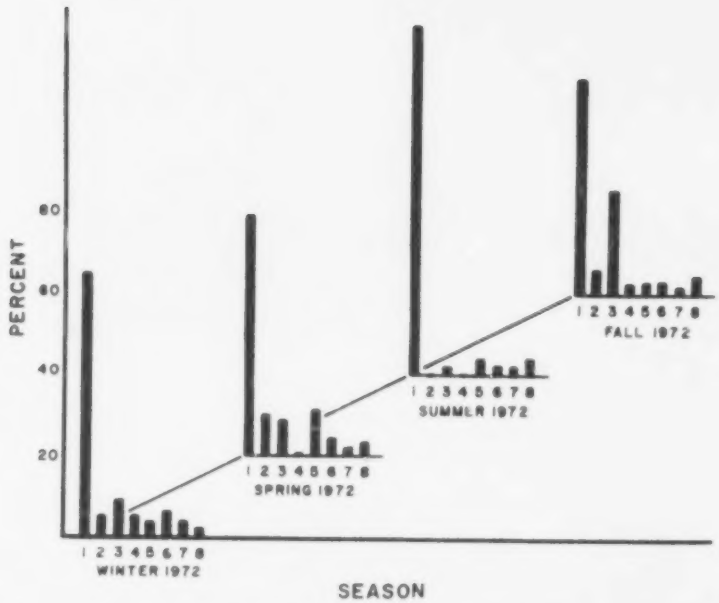

Figure 13. Seasonal population distribution of $HgCl_2$ resistant aerobic, heterotrophic bacteria. Five hundred and thirty-nine pure cultures found to be resistant to 6 ppm of $HgCl_2$ were collected from Stations B-1, B-2, A-1, A-2, and EB-1, and EB-2 during January 1972 through December 1972. "Winter" = 1/13/72 - 1/31/72, "Spring" = 3/29/72 - 6/1/72, "Summer" = 7/25/72 - 8/1/72, and "Fall" = 10/6/72 - 12/5/72. For generic key, see Fig. 12.

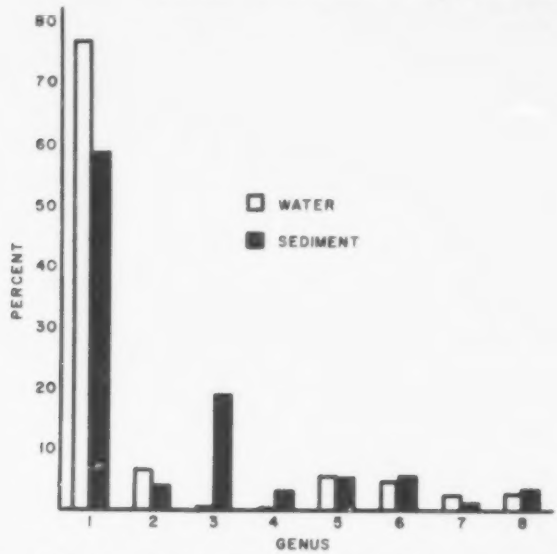

Figure 14. Mercury resistant bacterial population distributions in water and sediment in Chesapeake Bay. Three hundred and fity-six pure cultures found to be resistant to 6 ppm $HgCl_2$ were isolated during October 1971 through December 1973. The cultures were classified into genera or generic groups as listed in Fig. 12.

observed for the two Baltimore Harbor stations may be related to differences in hydrocarbon content (35), for it has been shown that mercury is associated with the hydrocarbon fraction of sediments in Baltimore Harbor. Although no attempt was made to characterize the chemical form of the mercury found in the sediments in Chesapeake Bay, apparent contrasts in bioavailability of mercury, as in the case of Baltimore Harbor, might well prove to be attributable to the concentration of oil in the sediment of a given site.

Relationship of Mercury Resistance and Metabolism

We have established previously that the effect of acquisition of resistance to a single mercury compound is pleiotropic, in terms of resistance to other mercury compounds (15). It was also shown that metabolic conversion of PMA to Hg^0 and benzene (1), an inducible mechanism, is associated with resistance (15). Similar inducible systems have been hypothesized for other organisms (11, 14). These findings concur with those relating to inorganic mercury and reported in this paper. Along this line of reasoning, the synthesis of methyl mercury, which also is a conversion of mercury to a more mobile state, is thought to be a resistance mechanism in Neurospora (35). The apparent adaptability of microorganisms to growth in the presence of mercury or mercury compounds suggests a possible causal relationship between mercury in the environment and the degree of biotransformation and mobilization of mercury. Our findings support the concept of a plate count index of potential in situ mercury metabolism. We have used concentrations of $HgCl_2$ and PMA (6 ppm and 3 ppm, respectively) routinely in preparing the media used in our investigation. These concentrations are considerably higher than the mercury concentrations encountered in Chesapeake Bay. However, our observations show that concentrations as low as 1.2 ppb of $HgCl_2$ or PMA will inhibit a measurable portion of the total population (34).

The phenomenon of bacterial reduction of Hg^{2+} is probably related to the evolution of Hg^0 from water and sediment which can be directly measured using fresh, raw sediment from Baltimore Harbor (F. Brinckman and W. Iverson, National Bureau of Standards, personal communication) and the Patapsco River (34) in a closed system fitted with a flameless atomic absorption spectrophotometer. Bothner and Carpenter (19) reported that total mercury concentrations in sediments of Bellingham Bay of Puget Sound, Washington, decreased according to first order kinetics, following the reduction of mercury discharges from a chlor-alkali plant. They found that the apparent half life of mercury in these sediments was 1.3 years. From data published by others, they also calculated half lives for the conversion of Hg^{2+} to methyl mercury to be 2.9 to 18 years. Our experiments, using similar concentrations of Hg and natural microbial populations isolated without selective enrichment for mercury resistance, also indicated a first order process (Table 17). An average half life of 12.5 days for Hg in aerated suspensions was calculated. It should also be noted that, in most cases, heat- or chloroform-sterilized inocula also induced a significant loss of Hg. The rate of loss observed in our experiments should be treated as an estimate of the upper limit

to be expected in the natural environment, since temperatures and cell concentrations used *in vitro* were much higher than measured *in situ*. However, the *in vitro* results clearly indicate a mechanism for the rapid mobilization of mercury in an estuary. The generation of Hg^0 may effect a net loss from the environment through volatilization, since Holm and Cox (38) have shown Hg^0 added to aqueous systems to be stable for periods up to 2 weeks.

Analysis of Seasonal Data

We have observed a seasonal fluctuation in numbers of mercury-resistant bacteria, and presumably mercury metabolism, and have attempted to relate it to various physical parameters. There are precedents for seasonal variations of marine bacteria, suggesting successions of (39, 40), but none more apropos than Jernelov's (41) observation of the seasonal changes in numbers of mercury-methylating bacteria in fish slime. Multiple regression analysis (Table 18) indicated that numbers of mercury-resistant bacteria are determined by different factors at each sampling area. A similar approach was employed by Brasfield (42) in her investigation of the incidence of pollution indicator bacteria.

Percentage resistance and mercury concentrations in sediments were positively correlated at the Annapolis and Eastern Bay stations, although not significantly at Annapolis. In contrast, a negative correlation was observed for samples collected in Baltimore Harbor, where Colgate Creek sediment contained higher levels of both Hg and petroleum. If mercury concentration is sufficient to specify bacterial population structure, then differences in the mercury-resistant populations of similar locales might reflect the proportion of total mercury which is biologically available.

Stations for comparative analysis were selected on the basis of similarities in sediment type and differences in mercury content. On this basis, data for Stations A-1 and EB-2 (sandy sediments) and B-1 were deleted from the regression equation. Percentage of mercury-resistant bacteria in the water column was found to be related to percentage of mercury-resistant bacteria in the sediment, suggesting a common determinant, most likely the mercury occurring in the sediments. The percentage of mercury-resistant bacteria in sediments was found also to be correlated with water transparency and dissolved oxygen.

A peak in sesten levels in the upper Chesapeake Bay coincides with the spring run-off from the Susquehanna River (32, 43). Smith has reported that most of the mercury in estuarine water is associated with the particulates (44). We have observed a negative correlation between water turbidity and presumptive *in situ* bacterial mercury metabolism, which may correspond to a decreased availability of mercury to the bacterial population. This process may be related to the adsorption of Hg to particulates. In fact, the binding of mercury and other heavy metals to organic suspended particulates, is the basis for their removal from water during sewage treatment (45, 46). Thus, the occurrence of a peak

in bacterial mercury metabolism in the spring is inconsistant with the elevated suspended sediment load normally present during that season. However, the lack of coincidence of the two peaks may explain the apparent paradox. In contrast, the elevated levels of dissolved oxygen during the spring season, in conjunction with organic complexing agents, may increase the solubility of Hg (23, 24).

Although analysis of variance for the multiple linear regressions for 3 stations and 6 stations gave significant F values (0.01 and 0.05 levels of significance, respectively), the multiple correlation coefficients (0.74 and 0.88, respectively) indicated that the parameters which were measured could not account for all of the variation in numbers of resistant bacteria. There are other seasonal influences in Chesapeake Bay which deserve mention but could not be considered in this study for lack of time, personnel and funds. For example, in Chesapeake Bay there is no spring phytoplankton bloom (47), but total nitrogen and zooplankton populations do peak during the spring months (32, 48).

The observed relationship between mercury-resistant bacterial populations and mercury concentration in sediment is compatible with the concept of bacterial "adaptation." However, because of differences in either the chemical form or the availability of mercury and the seasonal fluctuations in mercury-resistant bacterial population levels, a clear and unequivocal relationship cannot be established. That there is, indeed, a spring peak in the mercury-resistant bacterial population was confirmed by the monitoring data obtained during the first half of the second year. Unfortunately, because of the plan of work which had been laid out, the field work had to be terminated before the secondary fall peak could be monitored. The secondary peak in the mercury-resistant bacterial population which was observed in the Fall of 1972 may have been a consequence of the effects of tropical storm, Agnes, which occurred in June 1972. Correspondingly, data for the months preceding and following the storm suggest that there were significant, temporary changes in the bacterial flora following the storm (15).

Population Distribution

Using a taxonomic scheme similar to that employed in this study, Murchelano and Brown (40) obtained a seasonal distribution of heterotrophic bacteria in Long Island Sound which was much like that reported here for mercury-resistant bacteria in Chesapeake Bay. Total viable counts reached minima in the summer in both cases, and *Pseudomonas* spp. were predominant in Long Island Sound samples (40.6%), as in the case for mercury-resistant bacteria in Chesapeake Bay (66.0%). Our results and those of an earlier study of bacteria found in Chesapeake Bay water and sediment (49) suggest that the mercury-resistant bacterial population is richer in *Pseudomonas* spp. relative to the total population. Yet, the total number of *Pseudomonas* spp. did not correlate with seasonal mercury resistance (see Figs. 9, 10, and 13). However, when the *Pseudomonas* spp. were separated into physiological sub-groups, according to the method of Shewan et al. (27), it was evident that the proportion of

non-glucose-utilizing type III organisms correlated with average seasonal resistance (0.76 correlation). Interestingly, the two most metabollically active, Hg^{2+} reducing strains assayed in a survey of cultures (Table 14) were both type III Pseudomonas spp. In contrast, glucose oxidising Pseudomonas spp. types I and II declined in abundance from winter through the spring, summer and fall months.

Mercury mercaptide formation, via metabollically-generated sulfhydryl compounds has been shown to be a mechanism for mercury resistance in some microorganisms (50, 52). However, our data show that H_2S production is not an exclusive property of mercury-resistant bacteria, hence could not account for the level of mercury resistance in the bacterial populations studied. This particular point was carefully examined in order to establish the validity of a plate count index for potential mercury metabolism. Our conclusion was further supported by the fact that the seasonal distribution of H_2S-producing bacteria did not coincide with that of average mercury resistance in the viable, aerobic, heterotrophic populations in Chesapeake Bay water and sediment.

PMA was found to be considerably more toxic for the bacteria in Chesapeake Bay samples than $HgCl_2$. At 3 ppm, it was also more selective for Pseudomonas spp. When the PMA concentration was reduced to 0.3 ppm, inhibition of the total population was found to be comparable to that induced by the presence of 6 ppm of $HgCl_2$ in the plating medium. The relatively greater toxicity of organomercurials for microorganisms is a general phenomenon (34, 51, 52) and is very likely related to the lipophilic character of these compounds.

In summary, an ecological analysis of the distribution and function of mercury-resistant bacteria in Chesapeake Bay has been accomplished. The seasonal fluctuations in the numbers of these bacteria has been demonstrated and some of the influencing environmental factors elucidated. The cyclic oscillation observed in that physiological group of microorganisms comprising the mercury-resistant bacteria may be only a single component of a species succession. Work underway on the species composition of the total aerobic, heterotrophic bacterial populations in Chesapeake Bay should provide some further insight into this fascinating and heretofore undescribed aspect of bacterial population dynamics in nature.

SUMMARY

Total ambient mercury concentrations and numbers of mercury-resistant, aerobic, heterotrophic bacteria at six locations in Chesapeake Bay were monitored over a 17 month period. Mercury resistance expressed as the proportion of the total, viable, aerobic, heterotrophic bacterial population, reached a reproducible maximum in spring and was positively correlated with dissolved oxygen concentration and sediment mercury concentration and negatively correlated with water turbidity.

A relationship between mercury resistance and metabolic capability for

reduction of mercuric ion to the metallic state was established by surveying a number of $HgCl_2$-resistant cultures. The reaction was also observed in microorganisms isolated by differential centrifugation of water and sediment samples. Mercuric ion exhibited an average half life of 12.5 days in the presence of approximately 10^5 organisms/ml.

Cultures resistant to 6 ppm of mercuric chloride and 3 ppm of phenylmercuric acetate (PMA) were classified into eight generic categories. Pseudomonas spp. were the most numerous of those bacteria capable of metabolizing both compounds; however, PMA was more toxic and was more selective for Pseudomonas. The mercury resistant generic distribution was distinct from that of the total bacterial generic distribution and differed significantly between water and sediment, positionally, and seasonally. The proportion of non-glucose-utilizing mercury resistant Pseudomonas spp. was found to be positively correlated with total bacterial mercury resistance.

It is concluded from this study that numbers of mercury-resistant bacteria as established by plate count can serve as a valid index of in situ Hg^{2+} metabolism.

SECTION VI

EFFECTS OF MERCURIC CHLORIDE UPON GROWTH AND MORPHOLOGY OF SELECTED STRAINS OF MERCURY-RESISTANT BACTERIA

INTRODUCTION

Preceding sections of this report have included experimental evidence linking bacterial mercury resistance with *in situ* generation of Hg^0. The positive correlation observed between numbers of aerobic, heterotrophic bacteria resistant to given levels of mercury under laboratory conditions and ambient mercury concentration found in Chesapeake Bay sediments suggests that environmental mercury contamination can exert an effect upon the population structure of the estuarine microflora. To elucidate the mechanisms by which mercury manifests selective pressures under natural environmental conditions, we examined the effects of inorganic mercury upon growth and morpholoty of representative strains of bacteria isolated from Chesapeake Bay.

Investigations of mercury resistance in microorganisms have revealed several possible mechanisms for this resistance. Phytoplankton (53) and various bacterial strains (9, 12, 54) are capable of adapting to growth in the presence of mercury. In some bacteria, resistance is attained through the acquisition of plasmid, an extra chromosomal element which can be transferred intra- and inter-generically (5, 6, 7, 8, 9). Microorganisms detoxify mercury metabolically by formation of volatile Hg^0 (9, 12, 53, 54, 55), or of mercury mercaptides (50, 51).

Comparatively little information is available concerning the morphological effects of mercury upon microorganisms. Tingle et al. reported mitochondrial damage and swelling, pellicular membrane disruption, and loss of motility in *Tetrahymena pyriformis* incubated in a solution of 0.5 ppm of $HgCl_2$ (56). Exposure of cells of *Pseudomonas aeruginosa* to $HgCl_2$ caused swelling which was reversible by addition of sulfhydryl compounds (57). Also, extremely low levels of PMA (0.9 - 3 ppb) causing gross changes in the cellular form of growing cultures of *Phaeodactylum tricornutum* and *Chorella* have been described by Nuzzi (52).

The research work described in this report was carried out using cultures of bacteria isolated from Chesapeake Bay. In addition, the levels

of mercury employed in the study were selected so that correlation of results obtained with in situ conditions in Chesapeake Bay could be achieved.

MATERIALS AND METHODS

Organisms and Cultural Methods

Organisms were cultured in an artificial estuarine salts nutrient broth and agar medium described previously. (See Section V.) Cultures used in the study were isolated by spreading suitable dilutions of water and sediment on media supplemented with varying concentrations of $HgCl_2$ or PMA.

Electron Microscopy

Cells in late log phase cultures were transferred to fresh broth (0.1 ml/ml) in shaker flasks with and without sublethal concentrations of $HgCl_2$ added and were incubated at 25 C for 3 to 4 generation times, or until equal turbidities were obtained during early logarithmic phase of growth. The cultures were fixed according to the procedure described by Kellenberger and Ryter (58). Ten ml of culture was washed once in barbituric acid (K-R) buffer (pH 6.2), following which the cells were fixed in 1.0% osmium tetroxide in K-R buffer and prestained in 0.5% uranyl acetate, in K-R buffer, prior to stepwise dehydration through a 50%, 75%, 85%, 95%, and 100% ethanol and propylene oxide series. The specimens were embedded in Epon resin (Miller-Stephenson Chemical Co., Inc., Danbury, Conn.) (59), and ultra-thin sections were prepared using an LKB 800 Ultramicrotome III (LKB-Producter AB, Bromma 1, Sweden), fitted with a diamond knife (E. I. DuPont de Nemours and Co., Wilmington, Del.). The sections were placed on collodion-coated 200 and 300 micron mesh size copper grids and stained with a saturated solution of uranyl acetate (60) and lead citrate (61). An RCA EMU-3E or Hitachi HU-11A microscope (accelerating voltage of 50 kv) was used to view the sections.

^{203}Hg-labeled $HgCl_2$ Experiments

Culture 85, grown without mercury, was added to shaker flasks containing broth (0.1 ml inoculum/5 ml of broth). The first, i.e., control flask contained no mercury, the second flask, 2 or 4 ppm of ^{203}Hg-labeled $HgCl_2$ (approx. 200,000 dpm/ml). The flasks were incubated at 34 C (first experiment) or 25 C (second experiment) and optical densities relative to an uninoculated ^{203}Hg-labeled blank control were measured using a Klett-Sommerson Photoelectric Colorimeter (Klett Manufacturing Co., New York, N.Y.). Aliquots of cells were removed aseptically from the unlabeled medium and fixed for electron microscopy or diluted and plated on nutrient agar for viable counts. Duplicate 0.1 ml volumes of culture were removed at appropraite intervals for determination of total and non-filterable radioactivity by liquid scintillation.

Assay for Hg^{o} Production

The method of assay using ^{203}Hg-labeled $HgCl_2$ was as previously described. The assays were carried out at 25 C, using 1 ppm of $HgCl_2$.

RESULTS AND DISCUSSION

Survey of Morphological Effects

Representative cultures of bacteria isolated from colonies on agar media containing $HgCl_2$ or PMA were grown in broth containing sublethal concentrations of $HgCl_2$ in shaker flasks incubated at 25 C. The experimental cultures and controls without $HgCl_2$ were grown to early log phase and harvested. Thin sections were prepared from fixed and stained specimens, and the preparations were examined by transmission electron microscopy. Growth in the presence of mercury commenced after varying lengths of time, with an array of morphological effects which did not appear in control cultures of the same physiological age (Table 20). The most frequently observed defect involved the cell wall and cell wall synthesis. This was suggested by the formation of projections from the cell wall, elongated pleomorphic cells of the Gram-negative bacteria examined, and irregular cross-wall formation in the Gram-positive bacterial strains. A number of cultures also showed plasmolysis, indicating interference by the mercury with cytoplasmic membrane transport phenomena. These effects may be related to the finding that most of the mercury bound to bacterial cells has been found either on the cell wall or cytoplasmic membrane (this report, 10, 62).

Growth and Morphology of Representative Gram-positive and Gram-negative Species

Figure 15 shows growth of Bacillus strain 394 in the presence and absence of 5 ppm HgCl . After a one day delay ("lag") in the onset of growth, the cells were able to undergo logarithmic growth at a normal rate. A similar lag phase has been observed in the pattern of growth of other microorganisms in the presence of mercury (5, 9, 12, 53, 54, 63).

Table 20. SURVEY OF MORPHOLOGICAL EFFECTS IN SELECTED CULTURES OF BACTERIA GROWN IN THE PRESENCE OF $HgCl_2$[a]

Culture[b]		$HgCl_2$ concentration	Lag phase	
Genus	Strain	(ppm)	(h)	Morphological effects[c]
Pseudomonas	94	10	24	Cell wall outgrowth
Citrobacter	132	20	70	Plasmolysis, damaged membrane
Flavobacterium	119	10	40	Cell wall outgrowth, plasmolysis, elongated cells, irregular mesosomes
Vibrio	639	5	72	Giant, pleomorphic and rod-shaped cells, elongated cells
Bacillus	394	15	41	Irregular septum formation
Arthrobacter	72	5	24	Irregular septum formation
Enterobacter	85	1	0	Pleomorphic cells, outgrowth of cell wall, plasmolysis
Pseudomonas	B-16[d]	15	6 days	Small spherical cells bounded by common outer membrane
Pseudomonas	244	25	0	None

[a] Selected cultures were inoculated into shaker flasks of broth with and without $HgCl_2$ added and were incubated at 25 C until early log phase.

[b] Cultures were isolated and purified from colonies picked from mercury-containing nutrient medium inoculated with water and sediment samples collected in Chesapeake Bay and the Potomac River.

[c] Fixed and stained thin sections of cells grown, with and without $HgCl_2$, to the same cell density were compared.

[d] Supplied by R. A. MacLeod.

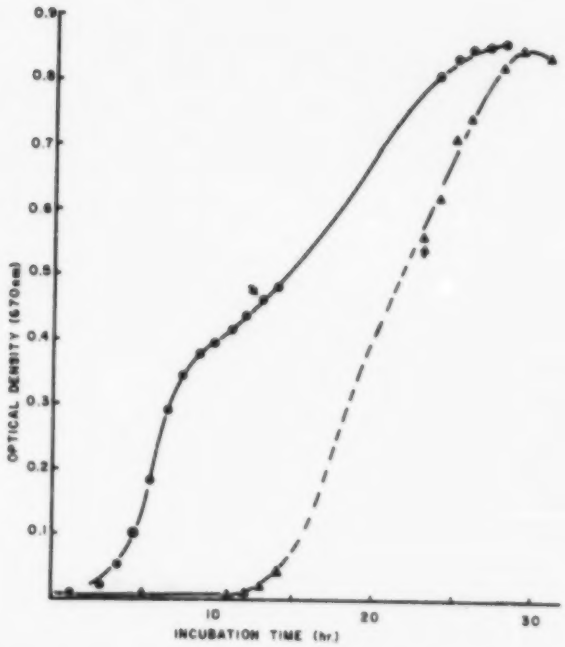

Figure 15. Growth of Bacillus, species 394, in the presence and absence of 5 ppm of $HgCl_2$. Broth in shaker flasks with (▲) and without (●) $HgCl_2$ was inoculated with overnight culture (0.1 ml/5 ml fresh broth). Growth was measured turbidimetrically during growth at 25 C. Samples were removed from each flask (arrows) for the morphological analysis of stained thin sections (see Figs. 15 and 16).

These cells showed irregular cross-wall formation and grossly deformed outer cell walls (Fig. 16). The effects were noticeably increased at a concentration of 15 ppm $HgCl_2$ (Fig. 17).

A Gram-negative species was selected for a more detailed investigation since Gram-negative organisms constitute 90% of the mercury-resistant bacteria isolated from Chesapeake Bay in the course of the study. The objective, in this instance, was to clarify events occurring while bacteria were apparently quiescent in mercury-containing broth and to relate these events to cytological changes associated with adaptation. Enterobacter, strain 85, was serially transferred several times in the absence of mercury, prior to inoculation into shaker flasks with and without 2 ppm of ^{203}Hg-labeled $HgCl_2$. After a 24 h lag phase, the mercury-containing culture grew at a rate comparable to that of the control culture (Fig. 18). Although the turbidity of the culture remained constant, a nearly 3-log drop in viable count occurred. Viable count and turbidity increased at the end of the lag phase. An isotope effect was suggested

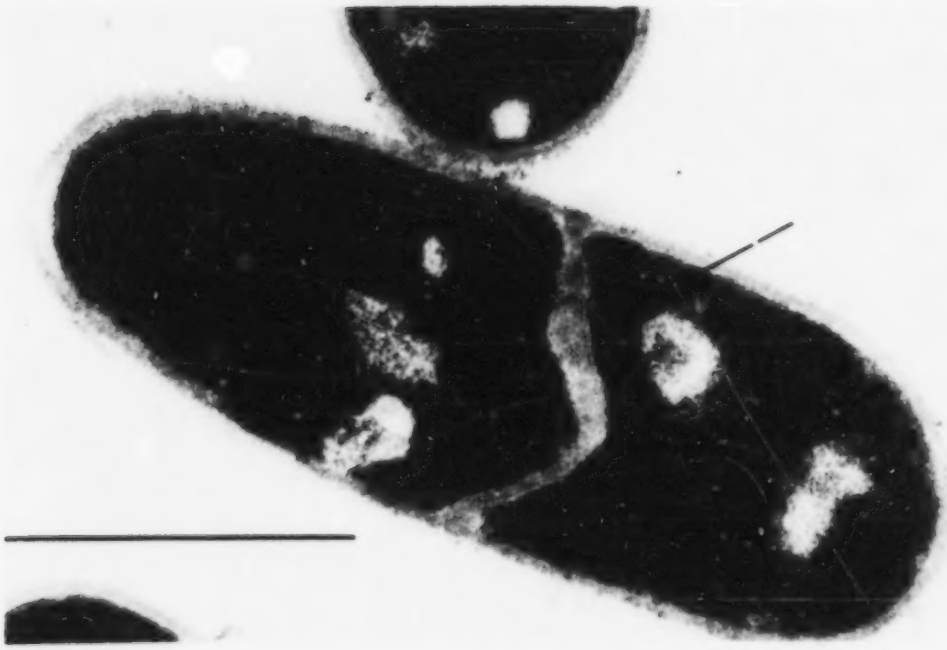

Figure 16. Thin section of cells of culture #394 grown in the presence of 5 ppm of $HgCl_2$. In comparison to cells grown without mercury, irregular septa were formed (arrow). Scale in this and subsequent micrographs is one micrometer.

Figure 17. Thin section of cells of culture #394 grown in the presence of 15 ppm of $HgCl_2$. Cells show irregular cell division and pleomorphism.

by the slightly shorter lag phase in the unlabeled mercury-containing culture. During the initial phase of incubation, radioactivity was rapidly lost from both the inoculated broth and from the sterile control.

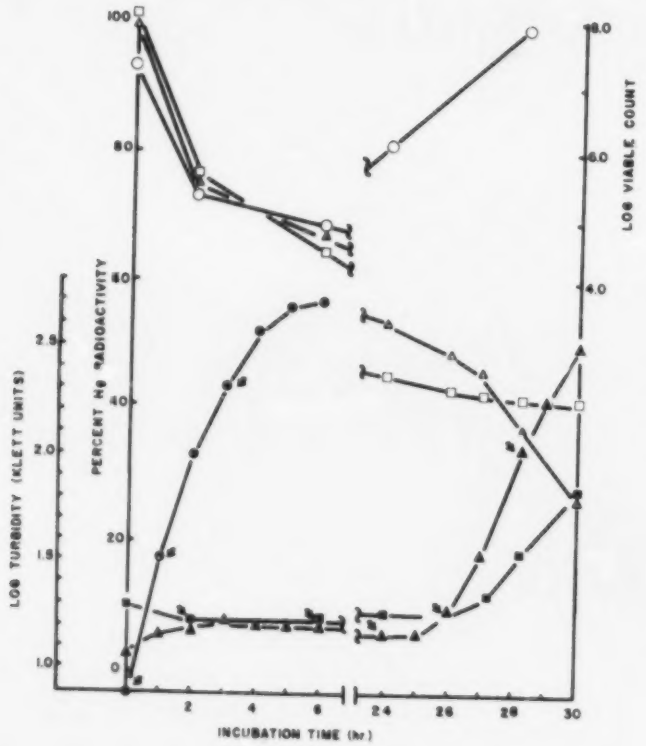

Figure 18. Growth and uptake and metabolism of mercuric ion by Enterobacter, strain 85. Shaker flasks containing broth with and without ^{203}Hg- and un-labeled $HgCl_2$ (2 ppm) were inoculated with an 18 h culture and incubated at 34 C. Turbidity of the culture without mercury (●) and the culture with labeled mercury (▲) were measured. Total (△) and non-filterable (■) activity of the latter were also measured. An uninoculated, Hg-labeled control flask (□) was also assayed for total radioactivity. Aliquots were removed from flasks without and with unlabeled mercury at intervals for examination by electron microscopy (arrows). Total viable counts of the unlabeled mercury culture (○) were obtained by spreading suitable dilutions upon basal nutrient agar.

Other investigators have shown that reducing agents such as glucose (64) and yeast extract (63) in growth media promote the reduction of Hg^{2+}. The loss of radioactivity from the control broth represented the volatilization of Hg as Hg^{0} and did not arise from adsorption to the glass

walls of the flask since only 8.9% of the radioactivity was recovered in sequential washes of the vessel using water, 0.5 N HNO_3, and 10% Radiac wash (Atomic Products Corp., Center Moriches, N. Y.). An initial, small loss of cell-bound mercury coincided with the drop in viable count, indicating lysis of the cells may have occurred. When the mercury concentration dropped approximately 45.0%, to 1.1 ppm, growth was initiated. At this point, the rate of loss of Hg from the broth accelerated in the inoculated flask, and the rate of cellular uptake, per unit volume of broth, increased. The possibility that the former effect resulted from biological reduction of Hg^{2+} was subsequently investigated.

After several hours of incubation, small colonial variants (85-S) of the $HgCl_2$-containing culture began to appear on the spread plates prepared for determination of total viable counts (Fig. 19). These colonies contained non-motile, pleomorphic cells, in contrast to the large colonies (85-L) comprising the remainder of the population.

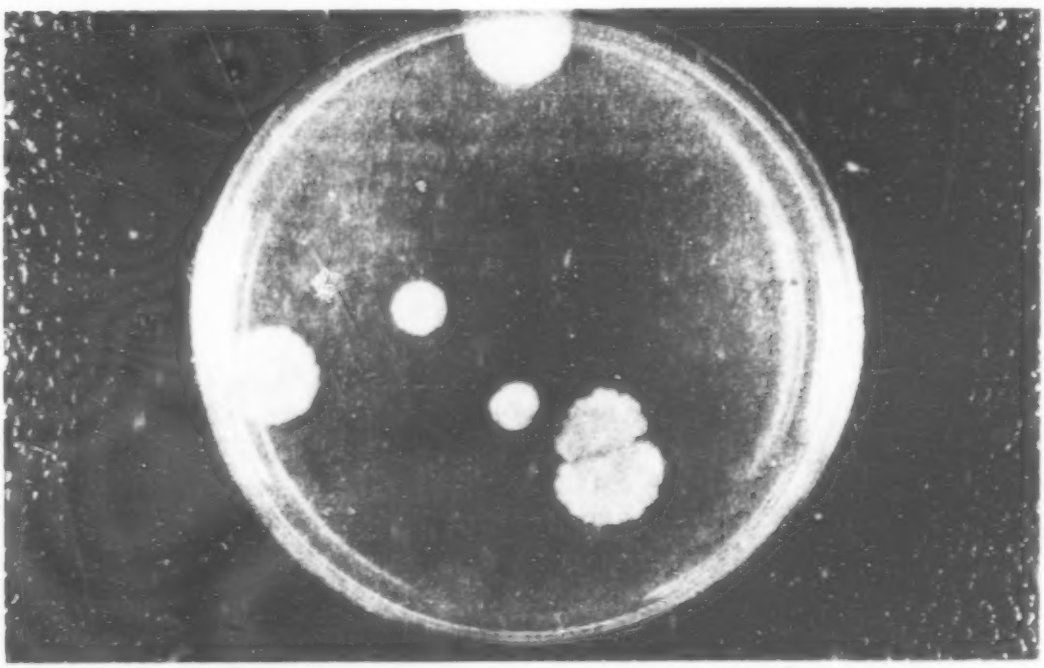

Figure 19. Colonial variation in Enterobacter, strain 85, grown in the presence of 1 ppm of $HgCl_2$. Samples of culture grown in 2 ppm unlabeled $HgCl_2$ containing broth were diluted and plated on basal nutrient medium (Fig. 17) and incubated at 25 C for 7 days. Small (85-S) and large (85-L) colonial forms were observed.

The experiment was repeated using an elevated concentration of mercury (4 ppm) to test the hypothesis that the length of the lag phase demonstrated by this organism was dependent upon reduction of mercury concentration to a specific threshold concentration. The patterns of growth and loss of mercury were as observed in the first experiment, except that the lag phase was found to be almost three days (Fig. 20).

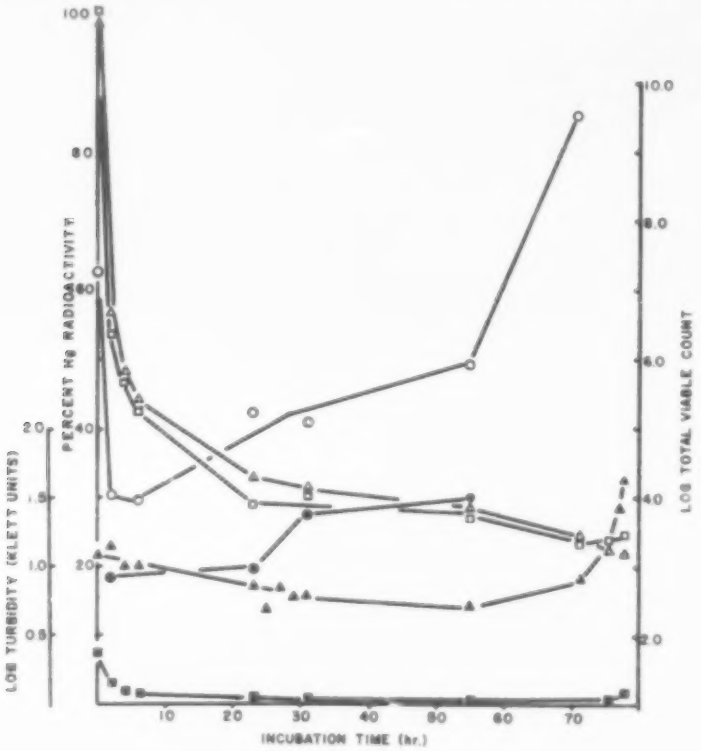

Figure 20. Growth and uptake and metabolism of mercuric ion by Enterobacter, strain 85. Shaker flasks containing broth with ^{203}Hg- and un-labeled $HgCl_2$ (4 ppm) were inoculated with an 18 h culture and incubated at 25 C. Turbidity (▲) and total (△) and non-filterable (■) radioactivity in the labeled culture were measured. An uninoculated, labeled control flask (□) was also assayed for total radioactivity. Total viable counts (○) and numbers of small colonial variants (85-S, Fig. 19) (●) were obtained by spreading suitable dilutions of unlabeled mercury containing medium upon basal nutrient agar.

However, during the lag phase, the mercury concentration dropped 70%, to a level of 1.2 ppm, a concentration closely approximating the concentration of 1.1 ppm $HgCl_2$ concentration at which growth commenced in the

first experiment. An increased isotope effect was observed in the second experiment, where the difference between the lag phase for labeled and unlabeled cultures was nearly 10 hours. Again, a drop in viable count was accompanied by a drop in cell-bound Hg. As in the first experiment, the rate of loss of Hg from the inoculated flask accelerated at the end of lag phase. Numbers of small colonial variants comprised approximately 1% of the total viable count, and these small colony variants increased in parallel with the total population (Fig. 20).

Fixed and stained thin sections prepared from cells harvested, in the first experiment, from unlabeled broth with, and without, mercury were examined. Control cells examined at the early, mid, and late logarithmic phase of growth were found to be normal in all respects, viz., array and morphology of the DNA fibrils, ribosomes and cell envelope, as well as mode of division. At 0 h, the mercury-containing culture was found to be identical to the 0 h control (Fig. 21). After 2 h (Fig. 22), the cells appeared normal in overall morphology. The DNA fibrils in some cells appeared condensed, rather than dispersed throughout the cytoplasm, and electron dense areas among the ribosomes were seen. Intact 6 h cells (Fig. 23), resembling 2 h cells, were accompanied by swollen and plasmolyzed cells. Approximately one-third of all the cells at this stage showed signs of lysis. At 24 h (Fig. 24), the culture was characterized by debris arising from lysed cells, as well as viable but pleomorphic cells demonstrating abnormal cross-wall formation. Electron dense clusters of ribosomes were more frequently seen in these cells. As growth progressed through the 26th and 28th hours, the cells became relatively more rod-shaped, although irregular cell walls and electron dense ribosome clusters were still present (Figs. 25, 26). The latter clusters are consistent with the preferential intracellular binding of Hg, but the technique of electron probe microanalysis, with sufficient resolution, would be required to clarify this point.

Properties of Mercury-selected Cultures

After repeated serial transfers in mercury-free medium, the small colonial variants lost their pleomorphism and regained motility, but retained their colonial morphology. If the mutants resulted as an "adaptation" to mercury, they should differ from the parent stock, with respect to mercury resistance and/or the ability to reduce Hg^{2+}. Comparative data on mercury resistance and ability to produce Hg from $HgCl_2$ of strains 85, 85-L and 85-S are presented in Table 21. It was evident that the isolates previously grown in mercury, specifically strain 85-S, were more resistant to mercury and demonstrated increased capability to metabolize Hg^{2+}. Thus, it appears that, during the lag phase of growth, mercury-resistant mutants are selected which are capable of releasing Hg^{o} from the growth medium. It is probable that these cells are identical to the pleomorphic, but viable, cells observed in thin sections of those cells examined after 24 h. When the mercury concentration was reduced to ca. 1 ppm via chemical and biological reduction of Hg^{2+}, the entire population initiated growth at a normal rate.

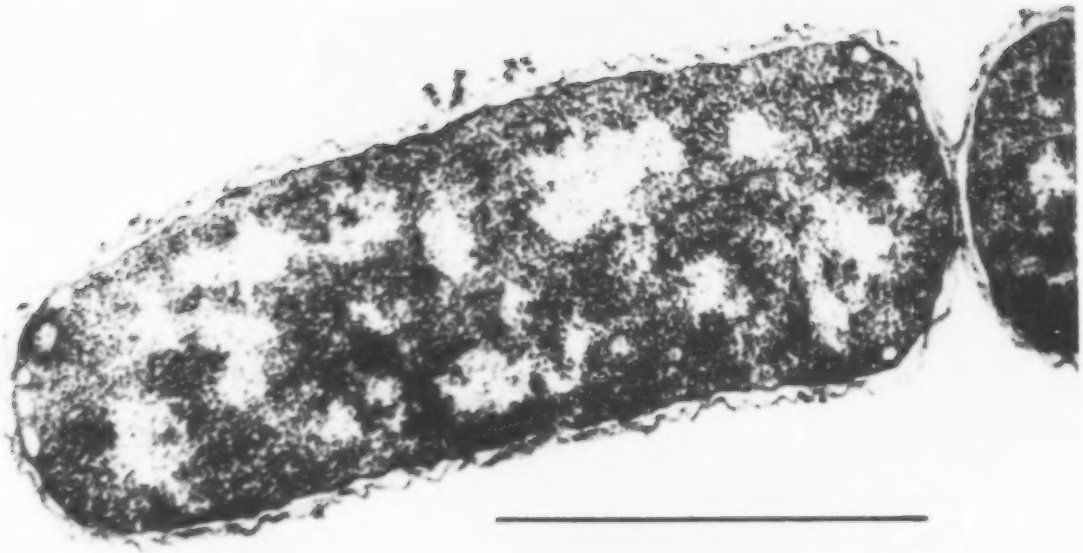

Figure 21. Thin section of culture #85 cells immediately after addition to broth containing 2 ppm of $HgCl_2$. The cells show dispersed chromatin, normal ribosomes and cell envelope constituents, as well as typical gram negative cell division by constriction.

Figure 22. Thin section of culture #85 cells after 2 h incubation in broth containing 2 ppm of HgCl . Similar in appearance to zero time, except for condensed chromatin, and the appearance of electron dense groups or ribosomes (arrow).

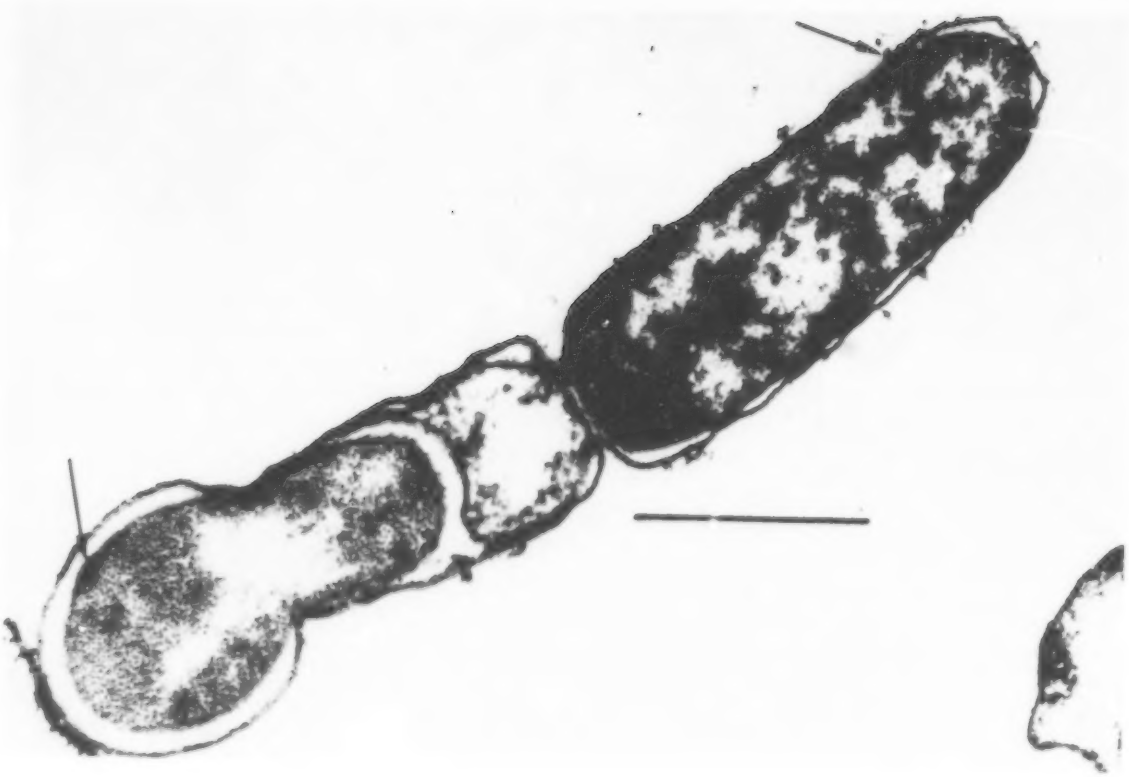

Figure 23. Thin section of culture #85 cells after 6 h incubation in broth containing 2 ppm of $HgCl_2$. The most prominent cytological features are plasmolysis and lysis in a large portion of the cells. Arrows indicate electron dense groups of ribosomes.

Figure 24. Thin section of culture #85 cells after 24 h incubation in broth containing 2 ppm of $HgCl_2$. Viable, pleomorphic cells and fragments of lysed cells are seen. Cellular division occurs by cross wall formation (arrows), rather than by normal constriction. Numerous electron dense groups of ribosomes are also seen.

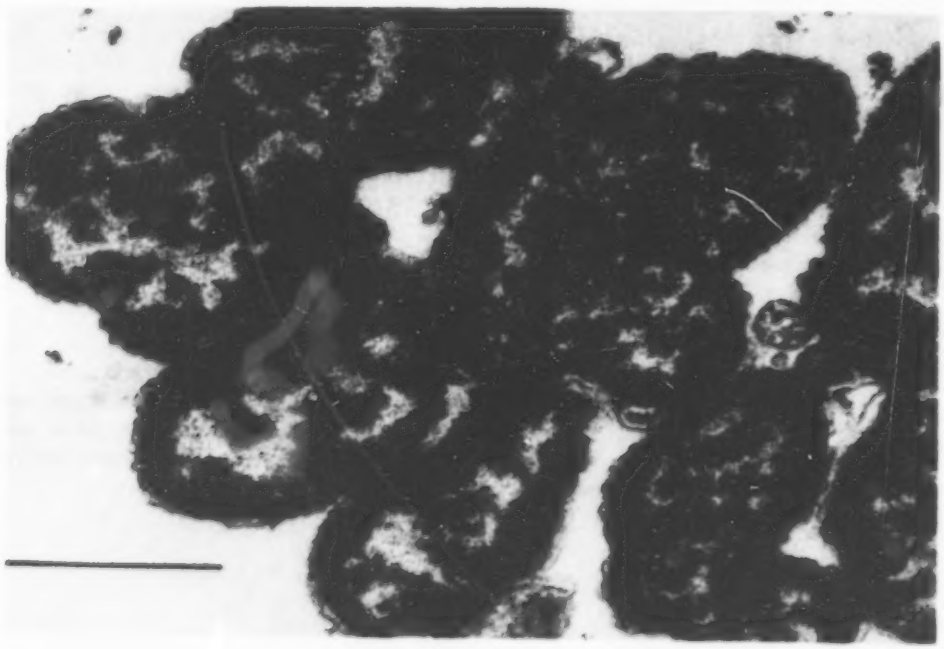

Figure 25. Thin section of culture #85 cells after 26 h incubation in broth containing 2 ppm of $HgCl_2$. Predominately viable, pleomorphic cells are seen. Electron dense clusters of ribosomes are present in all cells.

Figure 26. Thin section of culture #85 cells after 28 h incubation in broth containing 2 ppm of $HgCl_2$. The cells show reversion to rod-shaped morphology, although cell wall contours are still somewhat irregular. Electron dense clusters of ribosomes are present in fewer numbers.

Table 21. MERCURY RESISTANCE AND MERCURY METABOLISM OF STRAIN 85 BEFORE AND AFTER GROWTH IN THE PRESENCE OF $HgCl_2$[a]

A. Mercury resistance[b]

	$HgCl_2$ concentration (ppm)				
Culture[c]	2	4	6	8	10
85	+	+	-	-	-
85-L	+	+	+	-	-
85-S	+	+	+	+	+

[a]See the first experiment (Fig. 18).

[b]The cultures were serially subcultured in the absence of mercury. Each tube of mercury-containing broth was inoculated with 3 drops of a log phase culture and incubated, with shaking, at 25 C for 4 days.

[c]Culture 85 was the original inoculum, strain numbers 85-L and 85-S refer to small and large colonial types isolated during incubation of the $HgCl_2$ containing broth. (See Fig. 19.)

B. Formation of Hg^o[a]

		Percent decrease in ^{203}Hg radioactivity/60 min		
Culture	Dry weight (mg)	Live suspension	Heat killed[b] suspension	Net biological reduction[c]
85	2.05	9.8	16.1	0
85-L	1.50	7.1	3.6	3.5
85-S	1.90	11.3	3.0	8.3

[a]The assay was carried out at 25 C, with aeration, as described in Materials and Methods. The assay contained 1 ppm of $^{203}HgCl_2$ (approx. 120,000 dpm/ml).

[b]Sterile inocula were prepared by autoclaving at 121 C for 15 min.

[c]The difference in percent decrease between live and sterile inocula.

The *in vitro* experiments carried out in this study may be related to conditions in the natural environment. The positive correlation observed between mercury concentration and number of mercury-resistant bacteria in sediment suggests that selection does occur *in situ*. It is logical to propose that despite adverse effects of mercury or other heavy metals, microorganisms will prevail, in some form, because of their remarkable versatility. However, it is not yet possible to predict, quantitatively, the effects such a perturbation would have upon population structure in a natural microbial community or at the higher ecological level.

SECTION VII

THE ROLE OF BACTERIA IN THE MOVEMENT OF MERCURY THROUGH A SIMPLIFIED FOOD CHAIN

INTRODUCTION

Whether mercury levels in the oceans have risen significantly is a highly controversial issue (65, 66, 67). Undisputable, however, is the fact that marine animals are capable of concentrating mercury and other heavy metals to levels several orders of magnitude above that found in sea water (4), particularly in the proximity of known sources of pollution (3, 68). Regardless of the source, the consequences of the sequestration of mercury are serious because the highly toxic and persistent alkylated forms of mercury comprise most of the mercury in fish tissue (22, 69, 70). The mechanism(s) by which mercury is accumulated in higher organisms are not completely known. Barber et al. found no apparent relationship between mercury concentrations in marine fish and ambient sea water levels of mercury (66). Similarly, Windom et al. (71) found no significant differences in mercury concentrations in inshore and offshore catches of 35 species of North Atlantic fin fish. However, the amount of mercury accumulated in fish appears to be related both to the size (66, 70) and the species of fish involved (66). It is not known, as yet, whether given species of fish, such as tuna or swordfish, acquire relatively large amounts of mercury by unique mechanisms, which are species specific, or from participation in, or association with, a particular mercury-accumulating food web (72). Evidence both for (73, 74) and against (4, 69, 75) the magnification of mercury concentrations through trophic levels has been presented. The question has not been satisfactorily answered since none of the investigations were specifically undertaken to trace mercury flow through a specific food chain, under controlled conditions. Mercury uptake has been studied in individual marine animals such as the oyster (69, 76) and the fiddler crab (77), but in neither case were the microbiological parameters of the experiment measured or controlled.

We have investigated the ecology of mercury-resistant bacteria in Chesapeake Bay and have concluded that bacteria, by virtue of their ability to accumulate mercury and reduce Hg^{2+} to Hg^{o}, are influential in the mobilization and transformation of mercury in the Chesapeake Bay habitat. Consequently, an investigation of the role of bacteria in the

introduction of mercury into a simple estuarine food chain was initiated. Based upon a comparative survey of benthic and pelagic animals in Hawaiian waters, Klemmer and Luoma (73) concluded that mercury is probably most readily transported through short food chains directly linked to benthic organisms. After considering the available information and the fact that mollusks are known to accumulate mercury and other heavy metals (78), we selected for our study a food chain incorporating bacteria and a benthic filter feeder, the American oyster, Crassostrea virginica. A series of experiments were conducted to elucidate the routes and mechanisms of mercury introduction into aquatic food chains at the lowest trophic level.

MATERIALS AND METHODS

Aquarium System

The apparatus used in each of the three experiments which were carried out consisted of a flanged, 14 liter pyrex fermenter jar equipped with a stainless steel lid which was sealed with a rubber O-ring and fastened with bolts (Fig. 27). The lid had threaded nylon hose fittings wrapped with teflon tape to prevent leaks. A rubber serum bottle cap was placed on the central fitting to permit sampling of the closed system with a sterile 12 inch canula attached to a sterile syringe. A rack constructed from a glass rod was used to suspend the oysters off the bottom of the jar, above the teflon-coated magnetic stirring bar. A gas dispersion tube was connected to an outside air pump via Tygon tubing and a nylon hose fitting in the lid. The entire aquarium, complete with fiber glass air filter, was sterilized by autoclaving at 121 C for 15 min. Ten liters of 0.45 micron filter-sterilized, dilute, artificial sea water (Seven Seas Marine Mix, Utility Chemical Co., Paterson, N. J., 33% of sea water strength) were pumped in, and the aquarium was immersed in a 15 C constant temperature water bath. Circulation was provided by an external, submersible magnetic stirrer powered by water, and the water in the jar was oxygenated by air pumped through the fiber glass filter to the submerged gas dispersion tube. Effluent air from the aquarium was transported through Tygon tubing to two scrubbers in series, each containing 100 ml of $HgBr_2$ - KBr solution (see Section V) to trap volatilized mercury (Fig. 28).

Oysters and Bacteria

Oysters were dredged from Tolly Bar outside of Annapolis Harbor, Maryland, in March and April 1974, and maintained in raw bay water at *in situ* temperature and salinity until used in the experiments. The animals were scrubbed and placed in artificial sea water (approximately 12.0 °/oo salinity) at 6 C for approximately 1 week. The temperature of the water in which the oysters were held was brought up to 15 C gradually and was held for 3-4 days at 15 C prior to placing them in the sterile aquarium system. Bacterial cultures were grown in liquid basal medium (see previous sections) containing 6 ppm of $HgCl_2$. Fresh transfers of overnight cultures were grown for 3-5 h, centrifuged and resuspended in sterile salts solution, and added to the aquarium. Total viable bacterial counts

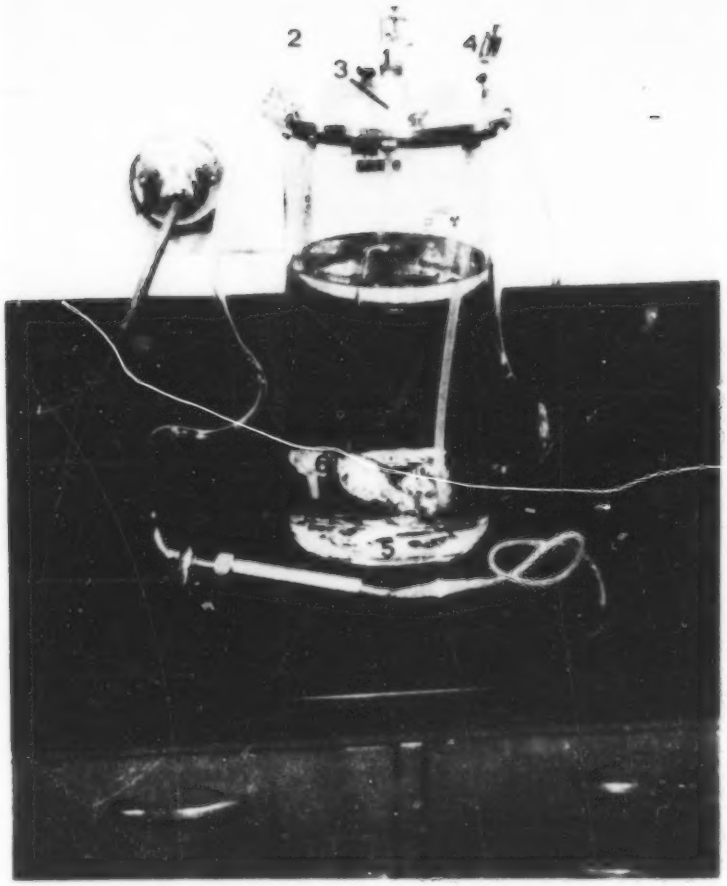

Figure 27. Sterilizable oyster aquarium. Sampling port with rubber septum (1); air inlet with sterile fiber glass filter (2); air outlet (3); water effluent (4); magnetic stirring bar (5); gas dispersion tube (6).

of aquarium water were determined periodically by spreading suitable dilutions of the water on solid basal medium and incubating the plates at room temperature for 2 weeks.

Experimental Procedures

Six oysters of uniform size were selected for each experiment. Two were shucked and freeze dried for total mercury analysis. The remaining four were placed in the sterilized aquarium. Bacterial suspensions were added through a port in the lid, followed by 1.0 ml of sea water containing

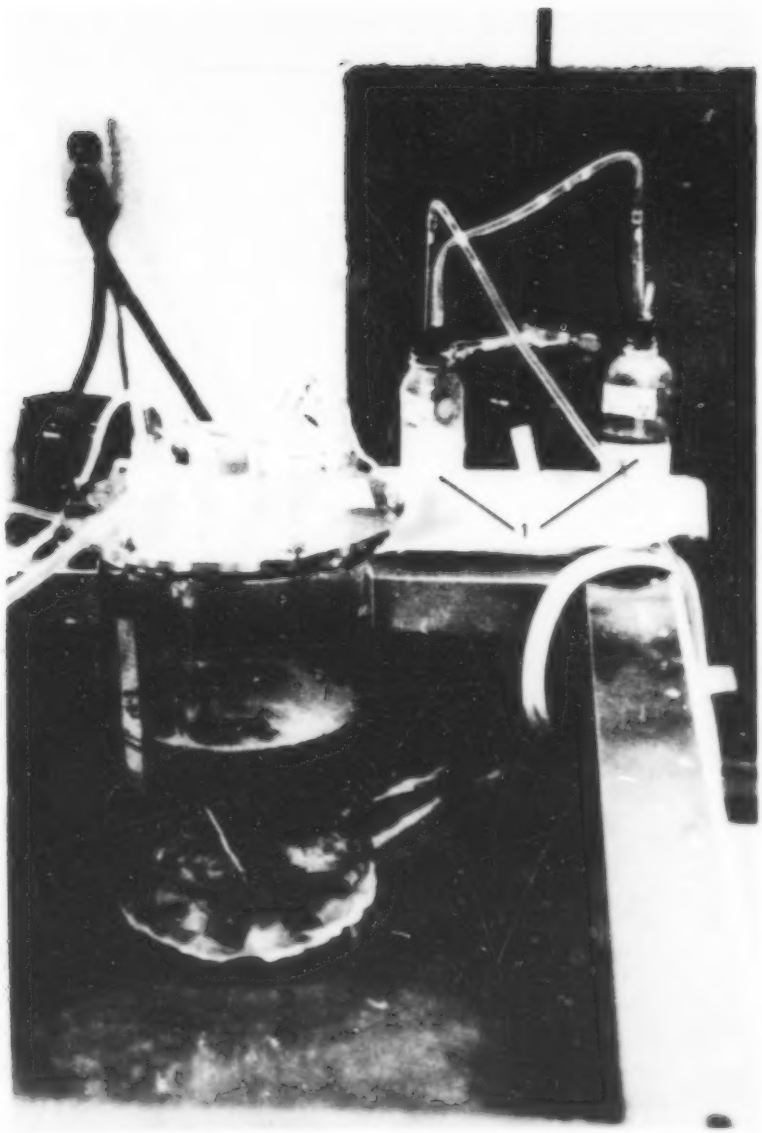

Figure 28. Oyster aquarium in operation. $HgBr_2$ - KBr traps (1); immersible, water propelled magnetic stirrer (2).

10-20 micro C of $^{203}HgCl_2$ to give a final concentration of 10.2 ppb. After an equilibration period of 10-15 min, duplicate 1 ml samples of aquarium water and scrubber solution were removed for scintillation counting. A second 1 ml sample of aquarium water was filtered through a 0.45 micron membrane filter, which was rinsed with three 1 ml volumes of artificial sea water. The filters were saved for scintillation counting. The above sampling procedure was repeated on four successive days. At the conclusion of the experiment, the oysters were removed from the aquarium, shucked, and dissected (Fig. 29). Tissues were rinsed in artificial sea water and placed in tubes for scintillation counting. Fecal material which had settled to the bottom was aspirated from the aquarium and collected on 8 micron membrane filters and rinsed in artificial sea water prior to scintillation counting. Radioactivity was measured in a Packard TRICARB Spectrometer equipped with an Auto-Gamma Spectrometer. The standard deviation was 1% of total counts for all oyster tissues.

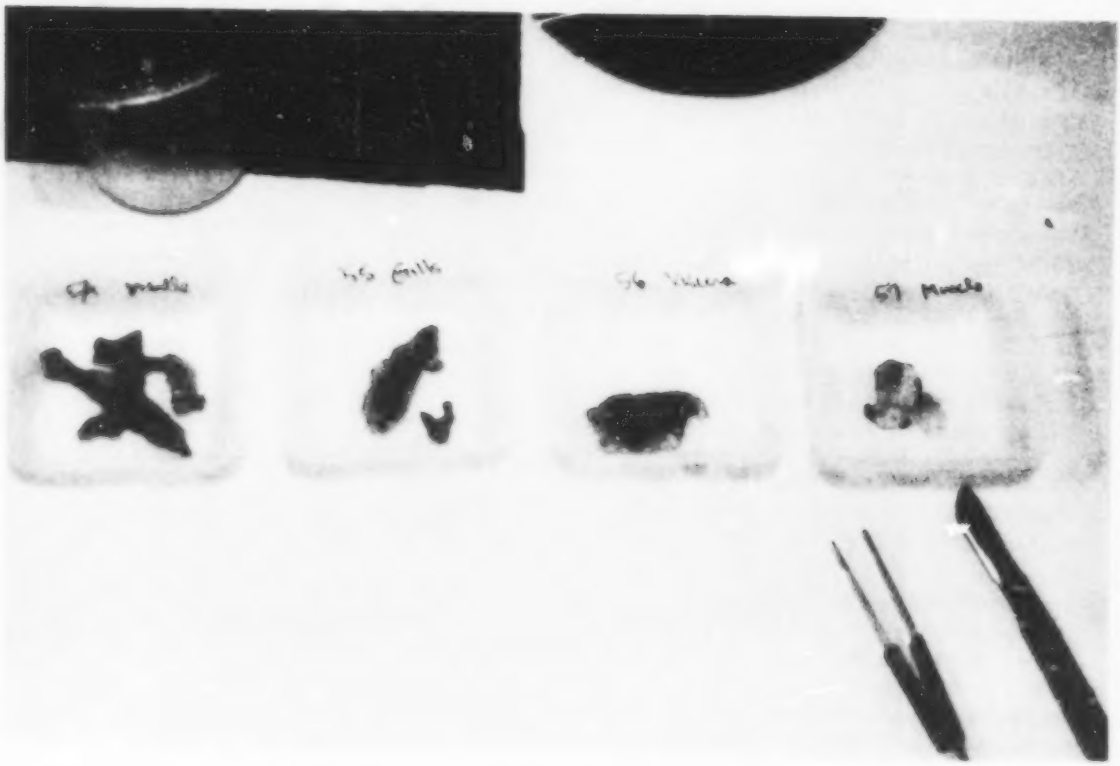

Figure 29. Oyster dissection. Oysters were shucked, and the mantle fluid was drained and retained. The oysters were dissected, and each of the tissues selected for analysis was rinsed in artificial sea water prior to measurement of radioactivity. Mantle, gills + labial palps, visceral mass, and adductor muscle (left to right).

RESULTS AND DISCUSSION

Three experiments, each using four oyster replicates per experiment, were completed (Table 22). The oysters were harvested at the same time of year from the same location. There was some variation in size, but it was determined that there was no correlation between size and the amount of mercury accumulated. Total mercury concentrations in the oysters prior to each experiment were nearly equal to the initial concentration of mercury in the aquarium water (Table 23). The significantly greater concentrations of mercury in treated oysters indicated that tracer uptake was truly a measure of accumulation and not exchange.

The purpose of the first experiment was to establish a base line for measuring the effect of Hg-resistant bacteria on Hg uptake by the oysters. In the second experiment, culture #14 (see Section V), which accumulates Hg (12 u gm of $HgCl_2$ accumulated/mg of cells/15 min from a solution of 6 ppm $HgCl_2$) was added. In experiment three, culture #5 (see Section V), which actively reduces Hg^{2+} to Hg^{o} (25.4 ug of $HgCl_2$ reduced to Hg^{o}/mg of cells/15 min from a solution of 6 ppm $HgCl_2$) was added. In experiments II and III, a quantity of cells approximately equal to that of the mixed flora in the aquarium was added.

Table 22. EXPERIMENTAL OUTLINE

	Oyster tissue wet weight (gm)[a]					
Experiment	Mantle fluid	Mantle	Gills	Visceral mass	Adductor muscle	Total
I[b]	12.3	5.7	5.0	7.6	3.6	34.1
II[c]	12.3	3.2	2.3	5.0	1.6	24.4
III[d]	15.3	2.7	1.9	4.1	1.2	25.2

[a] Average of four oysters.

[b] No bacteria were added. Total viable bacterial count increased from 3.4×10^4/ml to 2.5×10^5/ml after 4 days.

[c] Culture #14 added (1.2×10^9 cells). Total viable bacterial count increased from 7.2×10^4 to 9.6×10^4 after 4 days.

[d] Culture #5 added (1.2×10^9 cells). Total viable bacterial count increased from 1.0×10^5/ml to 1.4×10^5/ml after 4 days.

Table 23. AVERAGE TOTAL MERCURY CONCENTRATION IN UNTREATED OYSTERS[a]

Experiment	Total mercury concentration		Methyl mercury spike recovery (%)
	Dry weight (ppb)[b]	Wet weight (ppb)[c]	
I	324 ± 23	48.6	100
I	97 ± 14	14.5	109
II	201 ± 6	30.2	76
II	103 ± 25	15.5	83
III	239 ± 99	35.8	100
III	111 ± 17	16.7	90

[a]Two oysters were selected from each experimental group of six before treatment with mercury.

[b]Mean ± average deviation of three determinations.

[c]Extrapolated from dry weight concentration. Dry weight = 15% of wet weight (average).

The results of the three experiments are summarized in Table 24. Approximately twice as much mercury was accumulated by the oysters when mercury resistant bacteria were added to the aquarium (experiments II and III). The less-than-quantitative recovery obtained may have been a result of adsorption of mercury onto the aquarium and shell surfaces. Trapping of volatilized mercury by the first trap was essentially quantitative, with less than 0.10% of the total added radioactivity accumulating in the second trap. Approximately 10% of the added Hg was volatilized, even in the absence of added mercury metabolizing bacteria. The volatilization of Hg and the apparent excretion of Hg in fecal material suggest that the oysters, themselves, may actively metabolize Hg. During the course of each experiment, it was observed that the total concentration of mercury in the water approached the concentration of mercury in the microparticulate fraction of the water. Particulate mercury increased during the first 24 h and then decreased. During this period, the rate of decrease in dissolved Hg was most rapid.

The relative and absolute accumulations of mercury by individual oyster tissues are presented in Table 25. Generally, the largest proportion of the accumulated Hg was found in the gills (Table 25A). In experiments II and III, the amount found in the gills was statistically significantly greater than all other tissues examined. In the latter experiments, the mantle and visceral mass accumulated significantly greater proportions of Hg than either the adductor muscle or mantle fluid. Table 25B shows the tissue concentrations of mercury. Comparison of experiment I with

experiments II and III indicates that significant increases in mercury accumulation in gill tissue occurred when mercury-resistant bacteria were added to the system. The distribution of mercury in the tissues found in this study is consistent with the data published by others. Pentreath (79) showed that the stomach and gills were the primary sites of both concentration and exchange of heavy metal nuclides in the mussel, Mytilus edulis. Kopfler (80) reported that in oysters continuously exposed to 50 ppb of Hg as $HgCl_2$, the accumulation of Hg in gills was more than ten-fold higher than any other tissue. Mercury accumulated in the gills is probably entrapped in the microparticulate or dissolved form via the mucous secretions of the gills. Similarly, Pentreath found that ^{59}Fe was associated with the mucous covering of the gills (79).

The results of experiment III were unexpected, in that relatively less accumulation would have been predicted due to increased volatilization of Hg through bacterial metabolism. Although less Hg was accumulated than in experiment II, significantly more was accumulated than in the control experiment. The relatively high half saturation constant (20 ppm of HgCl) (see Section IV) for the evolution of Hg by the bacterial culture is a likely explanation. At the low concentration (10.2 ppb) of HgCl in the water, the bacterial cells will evolve Hg at a relatively low rate. However, culture #5 does accumulate Hg, albeit at 1/3 the rate of culture #14, and may also enhance Hg uptake by the oysters.

CONCLUSION

In short term experiments, mercury resistant, mercury accumulating bacteria caused relative increases in Hg accumulation in all of the oyster tissues examined. Increases in mercury concentration of the gills were statistically significant, with concentration factors in excess of three thousand. It can be concluded that bacteria may have a demonstrable effect upon mercury accumulation in those food chains which include filter feeding components.

Table 24. RELATIVE DISTRIBUTION OF MERCURY FOLLOWING EXPOSURE OF OYSTERS

	Percentage of total added mercury[a] after 4 days						
	Oyster						
Experiment	Uptake	Fecal material[b]	Adherent organisms[c]	Unfiltered water	Suspended particulates[d]	Volatile[e]	Recovery[f]
I	22.00	—	—	4.20	2.8	9.25	35.5
II	39.30	2.07	0.80	7.30	5.9	8.70	58.3
III	36.90	2.10	0.55	5.60	4.1	8.00	53.1

[a] The initial mercury concentration was 10.2 ppb of $HgCl_2$.

[b] Collected on 8 micron membrane filter after settling of aquarium contents.

[c] Worms and crustaceans adhering to oyster shells.

[d] Mercury retained by filtration through 0.45 micron membrane filters.

[e] Mercury accumulated in first $HgBr_2$ - KBr trap. Mercury accumulated in the second traps ranged from 0.03-0.09% of the total added mercury.

[f] Experiment I did not include fecal material or adhering organisms. Mercury adsorbed to aquarium and shell surfaces was not measured in any of the experiments.

Table 25. TISSUE DISTRIBUTION OF MERCURY AMONG TREATED OYSTERS[a]

A. Relative uptake

Experiment	Percent[b]					
	Mantle fluid	Mantle	Gills[c]	Visceral mass	Adductor muscle	Whole oyster
I	1.05 ± 1.43	14.51 ± 3.81	51.79 ± 11.63	29.57 ± 12.71	3.06 ± 1.11	4.19 ± 2.49
II	1.17 ± 0.52	12.14 ± 5.86	67.50 ± 9.36	16.87 ± 7.89	2.34 ± 0.51	9.84 ± 4.77
III	1.25 ± 0.54	20.67 ± 5.63	57.84 ± 5.44	17.70 ± 6.45	2.46 ± 1.20	9.20 ± 5.91

B. Mercury concentration[a]

Experiment	Mean mercury concentration (ppb $HgCl_2$ - wet weight)[d]					
	Mantle fluid	Mantle	Gills[c]	Visceral mass	Adductor muscle	Whole oyster
I	4.13 ± 5.69	162.10 ± 128.90	619.50 ± 474.40	231.90 ± 182.90	52.80 ± 44.10	173.30 ± 120.00
II	10.35 ± 8.59	397.05 ± 290.00	2974.10 ± 1703.70	338.30 ± 262.00	153.25 ± 105.76	424.60 ± 267.00
III	8.49 ± 6.23	714.78 ± 414.67	2778.65 ± 1060.78	408.77 ± 238.93	200.02 ± 197.69	389.77 ± 277.59

[a] After four days in the presence of $HgCl_2$ (initial concentration of 10.2 ppb).

[b] Tissues expressed relative to total uptake of $HgCl_2$ per oyster. For whole oysters, expressed relative to total initial amount of $HgCl_2$ added to the aquarium. Ninety-five percent confidence interval for mean of 4 determinations.

[c] Including labial palps.

[d] Ninety-five percent confidence interval for mean of 4 determinations. Underlined values are significantly different from control values (experiment I).

SECTION VIII

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SECTION X

GLOSSARY

Term	Definition
CFU	Colony-forming units
Hg^o	Elemental mercury
MeHg Cl	Methylmercuric chloride
PES	A 0.01 M phosphate-buffered "three salts solution" (pH 7.0)
PMA	Phenylmercuric acetate
Three salts	An artificial estuarine salt water mix of 1% NaCl, 0.23% $MgCl_2 \cdot 6H_2O$, and 0.03% KCl
TVC	Total viable, aerobic, heterotrophic count of bacteria by spread plate enumeration